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On the Relationship between Pantothenate Levels & the Growth Response of the Silkworm to Chloromycetin

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p-Aminobenzoic acid has been shown to annul the growth stimulatory effect of chloromycetin on silkworm. The beneficial influence of pantothenate in counteracting the growth depression caused by moderate levels of p-aminobenzoic acid has been revealed and the possibility of pantothenate ameliorating the toxicity due to chloromycetin has been suggested.

IN an earlier communication[1] it was reported that p-aminobenzoic acid (paba) was produced both *in vivo* and *in vitro* with chloromycetin in the silkworm, *Bombyx mori* L., and that whereas the antibiotic at low concentration promoted the growth of the larvae, paba, when fed at equivalent concentration, had a growth depressing effect. It was also shown that chloromycetin supplementation was followed by the excretion of paba, and it was suggested that excretion of paba occurs subsequent to its acetylation which is known to occur in the silkworm[2]. Under these circumstances, a relationship might be expected to exist between the growth response to chloromycetin and the coenzyme A and hence the pantothenate levels in the silkworm. It was also reasonable to expect that the pantothenate status of larvae would depend on the pantothenate levels in the feed. The purpose of the present study is to investigate both the effect of paba in aggravating the growth depression caused by high concentrations of the antibiotic and its reversal by pantothenate, and to show that a close relationship exists between the paba levels on the one hand and pantothenate on the other. In the light of the observations made, the dependence of antibiotic response on the pantothenate status of the insect has also been discussed.

Experimental procedure

The experiments were conducted with *Mysore* silkworms during the V instar. Ten replicates of two worms each were used for each treatment. About 0·2 ml. of the solutions was smeared on the mulberry leaves as uniformly as possible. When combination of the compounds had to be used, such as chloromycetin and paba, double strength solutions were prepared and mixed on the leaves themselves prior to feeding.

More growth experiments with paba-supplemented leaves were considered essential in the light of the report by Doskach[3] who found, in contradistinction to our own findings, that paba concentration of about 20 γ/worm/day influenced favourably the growth of the silkworm, especially of the weak larvae. Growth experiments were, therefore, repeated with paba using 100, 250 and 500 γ/ml. respectively. The lowest concentration, viz. 100 γ/ml., was estimated to procure a consumption rate of about 10-15 γ/day/worm.

The standard errors for all the growth measurements and the levels at which the values for the different treatments are significant with respect to the controls were calculated.

Results

The results presented in Table 1 clearly bring out the growth depressing effect of paba at the higher concentrations used. At 250 γ/ml. concentration the decrease in growth was significant at 0·1 level and at 500 γ/ml. concentration the decrease in growth was significant between 0·1 and 0·05 levels. This inhibition in growth could perhaps be attributed to the

rather large excess of paba fed (more than 20 γ/day/worm) as compared with the normal paba content (*c.* 0·5 γ). Also, the marked ability of pantothenate to overcome the inhibition (P = 0·05) was observed. In conformity with the observations of Doskach[3], the beneficial influence of paba on the weaker worms was also revealed from these experiments.

Reckoning both the growth depressing nature of paba in the silkworm and the ability of this organism to produce paba from chloromycetin, it was considered necessary to investigate the effect of adding (1) pantothenate and (2) paba to feeds containing different levels of chloromycetin in order to determine if pantothenate levels could modify the nature of response to chloromycetin, and, if so, whether paba production was the cause for this dependence. For this purpose, chloromycetin response was studied in the silkworm (1) during pantothenate deficiency induced by feeding the insect with ω-methylpantothenate and (2) when fed on normal mulberry diet supplemented with pantothenate.

The results recorded in Table 2 show that growth inhibition occurs in the larvae fed with ω-methylpantothenate at the concentration of chloromycetin at which accelerated growth was witnessed in the normal or the pantothenate supplemented larvae. It may also be noted that ω-methylpantothenate by itself, at the levels tried, does not inhibit the growth of the larvae. Perhaps a higher concentration might have induced growth inhibition since the metabolic analogue of pantothenate produces positive inhibition in the III and IV instar larvae at the concentration used in the present experiments.

A more detailed study of the relationship between pantothenate and chloromycetin was, therefore, undertaken. The results presented in Table 3 show that a comparatively large dose of chloromycetin was required to produce growth stimulation in the presence of pantothenate. In fact, at a concentration which normally was inhibitory (1000 μg.), there was a good growth response with pantothenate in the younger larvae. In the older larvae, on the other hand, chloromycetin at 100 μg./ml. concentration was not inhibitory. It would be of interest to carry out

TABLE 1 — EFFECT OF PABA ON THE GROWTH OF SILKWORM WITH AND WITHOUT PANTOTHENATE SUPPLEMENT

(*Growth values expressed in g./2 larvae 5 days after IV moult*)

	Paba conc., μg./ml.			
	Nil	100	250	500
Without Ca pantothenate	2·0339 ±0·0642	2·0405 ±0·0699	1·8892 ±0·0460	1·8550 ±0·0501
With Ca pantothenate (50 γ/ml.)	1·9239 ±0·0753	2·1544 ±0·0410	1·9289 ±0·0240	2·0353 ±0·0854

TABLE 2 — EFFECT OF PANTOTHENATE LEVELS IN THE GROWTH RESPONSE OF SILKWORM TO CHLOROMYCETIN

(*Growth values expressed in g./2 larvae 5 days after IV moult*)

Supplement	Water only	Chloromycetin (2 mg./ml.)	ω-Methyl-pantothenate (1 mg./ml.)	ω-Methyl-pantothenate (1 mg./ml.)+ chloromycetin (2 mg./ml.)	Pantothenate (50 μg./ml.)	Pantothenate (50 μg./ml.).+ chloromycetin (2 mg./ml.)
Larval weight	2·105±0·129	2·380±0·087	2·090±0·079	1·908±0·060	1·886±0·152	2·182±0·058
P	—	Between 0·1 and 0·05	—	0·1	—	Between 0·1 and 0·05

TABLE 3 — EFFECT OF PANTOTHENATE ON CHLOROMYCETIN RESPONSE

(*Growth values expressed in g./2 larvae*)

Days after IV moult		Chloromycetin conc., μg./ml.			
		Nil	300	600	1000
3	Without pantothenate	0·659±0·020	0·699±0·016	0·670±0·010	0·576±0·023
	With pantothenate (50 γ)	0·691±0·024	0·659±0·021	0·684±0·023	0·681±0·019
	P	—	0·15	*	0·01
6	Without pantothenate	1·190±0·0497	1·317±0·0383	1·256±0·0374	1·292±0·0183
	With pantothenate (50 γ)	1·357±0·0341	1·319±0·0355	1·285±0·0298	1·347±0·0237
	P	—	*	*	Between 0·1 and 0·05

*Not significant.

TABLE 4 — EFFECT OF PABA ON CHLOROMYCETIN RESPONSE IN THE SILKWORM

(*Growth values expressed in g./2 larvae 4 days after IV moult*)

	Chloromycetin conc., μg./ml.		
	Nil	500	1000
Without paba	1·787 ±0·077	1·757 ±0·061	1·692 ±0·053
With paba (100 μg.)	1·743 ±0·060	1·751 ±0·052	1·553 ±0·062
P	*	*	0·1

*Not significant.

studies with much higher doses of chloromycetin but the limited solubility of the antibiotic imposes a restriction on the operating range.

The additive effect of paba and of chloromycetin at higher concentrations was elucidated in another experiment. The results presented in Table 4 are in contrast with the observations of Smith[4] that the inhibition of growth in *Esch. coli* by chloromycetin was not affected by paba but was markedly reduced by *p*-nitrobenzaldehyde. This might be due to the fact that the former presumably does not stimulate growth of *Esch. coli* whereas the latter does.

Discussion

The fact that the stimulatory action of chloromycetin is counteracted by paba, which by itself depresses growth at moderate concentrations, brings to light the important role paba has in determining the nature of growth response by chloromycetin as also perhaps other aromatic amino or nitro compounds which might be formed from chloromycetin *in vivo*. Since the paba formed from chloromycetin in chloromycetin supplemented larvae is found to be excreted, it would appear that the realization of a positive growth response to the antibiotic is, at least partly, dependent on the efficiency of the organism to eliminate paba from the system. This efficiency is, in its turn, dependent on the pantothenic acid status of the silkworm and hence, ultimately, the type of response obtained with a known dosage of the antibiotic would be regulated by the pantothenic acid status of its feed. This has been clearly borne out by the results of the present study. The extreme variability observed in the response to the antibiotic in different experimental batches can perhaps be partially accounted for by the variability in the pantothenate level of the feed which would, in turn, modify the efficiency of the detoxicating system in the organism.

These results could also be of more valuable practical significance, when it is appreciated that pantothenate overcomes the inhibition due to large doses of chloromycetin. In therapeutic doses chloromycetin toxicity has been attributed to the nitrobenzene moiety of the molecule[5], and the fact that paba could be successfully counteracted by pantothenate is encouraging enough to warrant further exploration to see whether other toxic aromatic derivatives of chloromycetin could be detoxicated by pantothenate in a similar manner.

That streptomycin pantothenate is the least toxic among the salts of streptomycin has already been established[5]. However, the mechanism appears to be different in that the toxicity of streptomycin was reported to be due to the depletion of calcium through complex formation[6]; with streptomycin pantothenate complex formation was minimized and hence calcium was spared. In fact, calcium chloride was also found to counteract streptomycin toxicity[7]. In chloromycetin toxicity, there is reason to believe that pantothenate might help in eliminating toxic decomposition products of the antibiotic, and deserves further experimentation in other experimental animals.

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A Study of the Properties of Some Fungal Cellulases with Particular Reference to Their Inhibition

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The effect of various factors on the cellulase activity of culture filtrates of *Myrothecium verrucaria* and five jute-decomposing fungi grown on cellulose as the carbon source has been studied, using specially prepared swollen cellulose and carboxymethylcellulose as substrates. No cellulase is produced in the culture medium using glucose or sucrose as the carbon source. The relative cellulolytic potencies of the enzyme extracts bear no relation to the relative cellulose-decomposing capacities of the fungi. An acidic *p*H favours cellulase activity but the optimum varies with the substrate; the optimum temperature is *c.* 47°C. Cellulase in all filtrates is inhibited by heavy metals, *p*-chloromercuribenzoate and cystine, inhibition being prevented by cysteine and sodium sulphide, which indicates the presence of thiol groups essential for enzyme action. Acid dyes are inhibitory, and basic dyes stimulatory, at low *p*H, while at higher *p*H levels the latter are inhibitory.

THE cellulolytic function of microorganisms is performed through enzymes, collectively known as cellulase, secreted in considerable amounts by some fungi. As part of the problem of prevention of microbiological degradation of cellulosic materials, such as vegetable textiles, some properties of cellulases obtained from highly active fungi were studied, with particular reference to inhibition of enzyme activity.

Materials and methods

Cell-free culture filtrates of the following jute-decomposing fungi were used as source of enzyme:

	Lab. ref. No.
Curvularia lunata (Wakker) Boedijn	10
Aspergillus fumigatus Fresenius	14
Chaetomium indicum Corda	75
Penicillium rubrum Stoll	127
Penicillium wortmanni Klöcker	130.64
Myrothecium verrucaria (Alb. & Schw.) Ditm. ex Fr.	139

M. verrucaria has never been found on jute, but was included since its cellulase has been widely studied[1].

Each organism was grown on a sheet of Whatman filter paper No. 1 of 7 cm. diam., placed over a layer of glass beads, inside 250 ml. conical flasks, each containing 15 ml. medium. The medium used was Czapek-Dox salt solution (without Fe), containing 0·01 per cent yeast extract and of *p*H 5·4. After incubation at 30°C. for the required period, the contents of a flask were filtered through a Gooch crucible No. 4, the residue washed with 3 ml. water and the volume of the culture filtrate adjusted to the original volume of 15 ml. for use as enzyme solution. A minor modification was made in the growth medium of *P. wortmanni* 130.64 in that 0·1 per cent yeast extract was used in order to obtain regular and consistently good growth.

Two substrates, namely swollen cellulase[2] (SC) and sodium carboxymethylcellulose (CMC), were used for enzyme assay. Unless otherwise stated, the assay medium consisted of: 200 mg. substrate; 10 ml. of *M*/10 acetate buffer of *p*H 5·0 saturated with the antiseptic pentachlorophenol (Dowicide 7)[1]; 0·5 ml. of culture filtrate for CMC, 1 ml. for SC; the solution of any compound under test; and distilled water to give a final volume of 20 ml. The medium was taken in 125 ml. Pyrex conical flasks and incubated at 37°C. for 18 hr in the stationary state, since better results were obtained without shaking[3]. Hydrolysate fractions of 1 ml. for CMC and 5 ml. for SC were titrated for reducing sugar according to the micromethod of Somogyi[4]; the mean titre from duplicate flasks was taken as a measure of enzyme activity.

Results and discussion

The optimum conditions for enzyme production and activity are given in Table 1. The enzyme concentration, as determined by the extent of hydrolysis of both substrates, increases with *A. fumigatus* 14, *P. rubrum* 127 and *P. wortmanni* 130.64 up to the maximum period tested (14 days), but decreases after the 10th day with *C. lunata* 10 and *C. indicum* 75, and after the 8th day with *M. verrucaria* 139. It was decided to use filtrates of 8 days old cultures of this last fungus and 10 days old cultures of all the other organisms, since enzyme concentrations at these stages were not very different from the maxima. Interesting facts are noted when the potencies of enzymes present in the filtrates are compared with the cellulose-decomposing and jute-decomposing abilities of the respective organisms. Using these particular strains of fungi, among others, Basu *et al.*[5] studied their effect on the loss in strength of jute hessian, and Basu and Ghose[6] have estimated the weight lost, after growth, by filter paper pulp, jute cellulose and cotton cellulose (in the latter experiments, two other strains of *P. wortmanni* and not *P. wortmanni* 130.64 were used, and *M. verrucaria* was not tested). When arranged in decreasing order of activity the species occupy the following relative positions: with jute — *M. verrucaria* 139, *C. indicum* 75, *P. rubrum* 127, *P. wortmanni* 130.64, *A. fumigatus* 14 and *C. lunata* 10; with celluloses — *A. fumigatus* 14, *C. indicum* 75, *C. lunata* 10, *P. rubrum* 127 and strains of *P. wortmanni*. In the present case, when the highest enzyme titres obtained with SC within 14 days of incubation, as in Table 1, are considered, the relative positions are: *P. wortmanni* 130.64, *P. rubrum* 127, *A. fumigatus* 14, *C. lunata* 10, *M. verrucaria* 139 and *C. indicum* 75. This emphasizes the importance of extraneous factors in *in vivo* activity and it becomes evident that the cellulolytic capabilities of living organisms and of the extracted enzymes bear little apparent relation to each other. Basu and Ghose[7] have also observed that comparatively little enzyme could be detected in filtrates from several well-known cellulose decomposers, strongly degrading cellulose *in vivo*. Such differences may in part be connected with the impurity of the enzymes, since it has been shown that proteins and salts have a large effect on the activity of the cellulase of *M. verrucaria*[1,8].

The optimum *p*H for enzyme activity was determined by carrying out assays at *p*H values (maintained by *M*/10 acetate buffers) ranging from 3·8 to 5·8. The optimum *p*H is nearly always higher with SC as substrate. Similarly, the optimum temperature for enzyme activity was determined by carrying out assays at temperatures ranging from 32° to 52°C.; the optima are the same for both substrates. Only *Aspergillus fumigatus* 14 shows increasing enzyme activity up to the maximum temperature tested. This organism is also known to grow better at a higher temperature than is usual with other fungi.

All these fungi were found unable to secrete cellulase when grown on glucose or sucrose, thus confirming their adaptive nature.

Inhibition and stimulation of activity for the enzyme of *C. lunata* 10 are shown in Table 2. Other enzymes reacted qualitatively in the same manner. The inhibitors are much less effective with CMC than with SC. For any inhibitor, the degree of inhibition is not necessarily proportional to its concentration; mercury is more inhibitory than copper. The most potent cellulases, namely those from *P. rubrum* 127 and *P. wortmanni* 130.64, were also found to be the most resistant towards inhibitors, while the weakest cellulases (from *C. indicum* 75 and *M. verrucaria* 139) were the least resistant, the others occupying intermediate positions.

While Cu^{++} and Hg^{++} may inhibit by combining with polar groups like —COOH and $—NH_2$ in the enzymes, cystine and PCMB are believed to react with thiol groups through oxidation and mercaptide

TABLE 1 — OPTIMUM CONDITIONS FOR ENZYME ACTIVITY

Enzyme source	Incubation period for fungus *days*		*p*H for enzyme activity		Temp. for enzyme activity °C.	
	CMC	SC	CMC	SC	CMC	SC
C. lunata 10	10 (3·60)*	10 (2·50)*	5·2	5·8	47	47
A. fumigatus 14	14 (6·60)	14 (3·64)	4·8	5·2	52	52
C. indicum 75	10 (3·95)	10 (1·35)	5·2	5·8	42	42
P. rubrum 127	14 (6·85)	14 (6·05)	4·4	5·2	47	47
P. wortmanni 130.64	14 (7·55)	14 (6·70)	4·4	5·2	47	47
M. verrucaria 139	8 (3·30)	8 (1·75)	5·8	5·8	42-47	42-47

*Values given in parentheses in these columns refer to titre in ml.

TABLE 2 — INHIBITION AND STIMULATION OF ENZYME ACTIVITY

(*C. lunata* 10)

Enzyme activity with heavy metals and thiol reagents			Enzyme activity with inhibitors and reversing agents					Enzyme activity with acid and basic dyes		
Inhibitor	Conc. *M*	Mean activity %	Inhibitor	Conc. *M*	Reversing agent	Conc. *M*	Mean activity %	Dyes (conc. $10^{-4}M$)	*p*H	Mean activity %
CMC AS SUBSTRATE										
$CuSO_4.5H_2O$	10^{-3}	92·6	$CuSO_4.5H_2O$	5×10^{-3}	—	—	68·0	Congo red (acid)	3·2	14·9
	5×10^{-3}	46·3		5×10^{-3}	Cysteine-$HCl.H_2O$	$2·5\times10^{-2}$	163·8		6·0	85·4
	10^{-2}	34·5								
$HgCl_2$	10^{-4}	96·3	$HgCl_2$	5×10^{-3}	—	—	61·1	Erythrosin (acid)	3·2	24·3
	10^{-3}	39·9		5×10^{-3}	Cysteine-$HCl.H_2O$	$2·5\times10^{-2}$	348·6		6·0	88·5
PCMB	10^{-3}	39·9	PCMB	5×10^{-3}	—	—	61·1	Crystal violet (basic)	3·2	209·0
	2×10^{-3}	30·6		5×10^{-3}	Cysteine-$HCl.H_2O$	$2·5\times10^{-2}$	415·2		6·0	100·0
				5×10^{-3}	Na_2S	$2·5\times10^{-2}$	126·3			
l-Cystine	2×10^{-3}	83·5						Bismarck brown (basic)	3·2	390·1
	5×10^{-3}	63·5							6·0	103·2
SC AS SUBSTRATE										
$CuSO_4.5H_2O$	10^{-3}	62·9	$CuSO_4.5H_2O$	2×10^{-3}	—	—	16·6	Congo red (acid)	3·2	85·7
	10^{-2}	11·0		2×10^{-3}	Cysteine-$HCl.H_2O$	10^{-2}	416·6		6·0	107·7
				2×10^{-3}	Na_2S	10^{-2}	72·9			
$HgCl_2$	10^{-4}	26·1	$HgCl_2$	2×10^{-4}	—	—	61·0	Erythrosin (acid)	3·2	85·7
	10^{-3}	0·8		2×10^{-4}	Cysteine-$HCl.H_2O$	10^{-3}	159·0		6·0	54·8
PCMB	2×10^{-4}	12·5	PCMB	2×10^{-4}	—	—	6·3	Crystal violet (basic)	3·2	114·2
	5×10^{-4}	4·2		2×10^{-4}	Cysteine-$HCl.H_2O$	10^{-3}	141·6		6·0	135·3
				2×10^{-4}	Na_2S	10^{-3}	29·2			
l-Cystine	5×10^{-4}	84·8						Bismarck brown (basic)	3·2	685·7
	10^{-3}	51·9							6·0	140·7

formation respectively. More positive indication of the presence of active thiol groups was sought by the use of cysteine and sodium sulphide, to see if inhibition could be counteracted, apparently with the regeneration of these groups. The results presented in Table 2 show that inhibition is not only reversed but even activation takes place. It is known that cellulase can be stimulated[8], e.g. by proteins at *p*H values 4·8 and 5·3. On the whole, the presence of essential thiol groups in the enzymes is indicated.

The effect of two acid dyes, Congo red and Erythrosin, and two basic dyes, Crystal violet and Bismarck brown, studied at *p*H values 3·2 and 6·0 shows that basic dyes cause enzyme stimulation and acid dyes inhibition at *p*H 3·6, whereas at *p*H 6·0 the situation is reversed. These results agree with those of Basu and Whitaker[1], obtained with the enzyme of *M. verrucaria*.

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Mode of Action of Psoralen in Pigment Production: Part I—Action of Ultraviolet Radiation on Psoralen

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Irradiation of psoralen in suspension or aqueous ethanol solution with ultraviolet radiation (maximum emission at 253·7 mμ), with or without cysteine or glutathione, has been shown to produce fluorescent products other than psoralen. The ultraviolet absorption spectrum of the major product (R_f 0·62-0·67) has been found to be different from either furocoumaric acid or dimer of psoralen. Incubation of psoralen with cysteine or glutathione at acid *p*H likewise produced other fluorescent products. The possible significance of such transformations of psoralen in pigment production has been discussed.

EXPOSURE to ultraviolet light has been shown to increase melanin formation in human skin. Further potentiation of this effect has been observed by the *in vivo* administration of certain furocoumarins[1], e.g. psoralen and 8-methoxy psoralen (8-MOP), but the actual mechanism by which this effect is produced is not clearly understood. However, it has recently been shown that measurable amounts of intraperitoneally administered 8-MOP (or its metabolites) appear in the skin and brain tissue of albino mice[2]. *In vitro* photo-oxidation of 3,4-dihydroxyphenyl alanine (DOPA) to melanin has also been shown[3] to be brought about by 8-MOP. Further, it has been suggested[4,5] that the sulphydryl groups present as normal inhibitors of tyrosinase activity in the skin are removed or inactivated by ultraviolet radiation, thereby increasing melanin formation. It was, therefore, considered of interest to investigate the action of ultraviolet radiation on psoralen under different conditions and in the presence of compounds containing sulphydryl groups. The results of these studies are reported in the present communication. The ultraviolet absorption spectra of psoralen and some of the products obtained after irradiation have also been presented.

Experimental procedure

Irradiation of psoralen — Suspensions of finely powdered psoralen* (3 mg. in 3 ml. water or buffer) were irradiated with an ultraviolet lamp (Hanovia Chromatolite, 30; maximum emission at 253·7 mμ) kept at a distance of 9-10 cm. from the surface of the suspension. In one experiment irradiation of psoralen was also done with β-rays from P^{32} as orthophosphate. The petri dish was covered with tin foil on the sides to avoid radiation loss.

Chromatographic separation of products obtained by irradiation of psoralen — In view of the difficulties experienced by early workers[6-10] in the chromatographic separation of furocoumarins and the non-resolution and diffuseness of bands on unbuffered Whatman No. 1 paper strips, separations in the present studies were carried out in the descending manner on paper strips buffered with 0·1*M* Na_2HPO_4, using the organic phase of *n*-butanol saturated with water (4: 1) as the developing solvent. The supernatant solutions after irradiation of psoralen or other mixtures were applied as bands and, after development, the bands were examined under ultraviolet light. The fluorescent bands were marked, eluted with water or ethanol (95 per cent vol./vol.) and the absorption spectra of these eluates were obtained with the eluant as blank, after suitable dilution, using the Unicam spectrophotometer.

Results

The R_f values of products obtained by irradiating psoralen under different conditions are recorded in Table 1. It is seen that under the conditions of irradiation used in the present experiments, psoralen gives rise to products having different R_f values. The major product obtained on irradiation

*Psoralen was extracted and purified from *Psoralea corylifolia* in the Medicinal Chemistry Division of this Institute.

of psoralen, either in water suspension or aqueous ethanol or as a band, had R_f values of 0·63, 0·64 and 0·76 respectively. The absorption spectrum (Fig. 1) of the eluate of this product indicates a generalized absorption which becomes stronger towards the shorter wavelength region. No distinct maxima were obtained between 220 and 390 mμ, as against the usual three maxima observed with psoralen. This compound also appeared to be different from the dimer or furocoumaric acid[11]. The absorption spectrum of the aqueous supernatant of irradiated psoralen was, however, similar to that of psoralen.

The data recorded in Table 1 also show that irradiation of psoralen in the presence of an aqueous solution of cysteine hydrochloride produces a number of fluorescent products with the R_f values 0·04, 0·19, 0·41, 0·59 and 0·67, besides that of psoralen (R_f 0·94). The psoralen control eluted from buffered paper had maxima at 245, 290 and 330 mμ. The absorption spectra of the fluorescent products likewise had no maxima between 220 and 390 mμ and showed a generalized absorption which became stronger towards the shorter wavelength region.

Incubation of psoralen with aqueous cysteine hydrochloride (1 mg./ml.) for 24 hr or longer at 37°C. (*p*H of resulting solution 2-3) was also found to produce a number of fluorescent compounds with R_f values 0·028, 0·20, 0·53 and 0·67, besides the psoralen band (R_f 0·94). The formation of these products was found to depend on *p*H, time and temperature of incubation. Incubation of psoralen with aqueous cysteine hydrochloride at 25°C. for 4 hr did not produce these products; incubation for 24 hr, however, produced only one compound with R_f value 0·67. The other products could be formed in a shorter time by incubation at 60°C. and also by increasing the amount of added cysteine hydrochloride. Incubation of psoralen with glutathione (1 mg./ml.) at 25°C. for 24 hr was also found to produce the band with R_f value 0·66 along with the band for psoralen. The ultraviolet absorption spectrum of the eluate of compound with R_f value 0·67 produced by incubating psoralen and aqueous cysteine hydrochloride also showed the same pattern of absorption as described earlier. Psoralen incubated in a solution of dilute hydrochloric acid of the same *p*H as that of cysteine hydrochloride and for the same period or incubated with cysteine hydrochloride in phosphate buffer (*p*H 7·4 or higher) did not produce any of these products. That these products are not the reduction products of psoralen was demonstrated by incubating psoralen with aqueous sodium hydrosulphite solution for 72 hr, when none of the above products was formed. Irradiation of psoralen, however, in the presence of aqueous sodium hydrosulphite, brought about the formation of a number of fluorescent bands whose R_f values are given in Table 1. Irradiation of psoralen with β-rays from P^{32} orthophosphate or with a 100 W. electric bulb did not produce any of the products.

TABLE 1 — R_f VALUES OF PRODUCTS PRODUCED BY ULTRAVIOLET IRRADIATION OF PSORALEN UNDER DIFFERENT CONDITIONS

(*Irradiation in all experiments was for 6 hr at 37°C.*)

Form in which psoralen was irradiated	Mean R_f values of fluorescent products								
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
Ethanol: water suspension (2:1 or 1:1)	0·00 (++)*	—	—	0·44 (+)	—	0·63 (+++)	—	0·83 (++)	0·93 (+++)
Water suspension	0·00 (++)	—	—	0·43 (+)	—	0·64 (+++)	—	0·84 (+)	0·93 (+++)
As a band on buffered paper	0·00 (+++)	0·19 (+)	—	0·41 (++)	—	—	0·76 (++)	—	0·92 (++)
0·1*M* phosphate buffer (*p*H 7·4) suspension	0·03 (++)	—	—	—	0·58 (++)	—	—	—	0·92 (++)
Dil. HCl suspension	—	—	—	—	0·60 (++)	—	—	0·83 (+)	0·93 (+++)
0·1*M* phosphate buffer (*p*H 8·5) suspension	0·00 (+)	—	—	0·41 (+)	0·60 (++)	—	—	—	0·93 (++)
Water suspension+ cysteine HCl (1 mg./ml.)	0·04 (++)	0·19 (+)	—	0·41 (+)	0·59 (++)	0·67 (+++)	—	—	0·94 (++)
Water suspension+ cysteine HCl (1 mg./ml.) buffered *p*H 7·4	0·03 (+)	—	—	0·42 (++)	0·60 (++)	—	—	—	0·94 (++)
Water suspension+ glutathione (1 mg./ml.)	0·01 (+)	0·16 (+)	0·28 (+++)	0·45 (+)	0·58 (+++)	—	0·71 (+++)	—	0·93 (++)
Water suspension+ $Na_2S_2O_4$ (1 mg./ml.)	0·03 (+)	—	0·37 (+)	0·45 (+)	0·54 (+++)	0·62 (+++)	—	0·81 (+)	0·93 (++)

*The intensity of fluorescent bands given in parentheses is indicated as follows: +, faint; ++, medium; +++, strong.

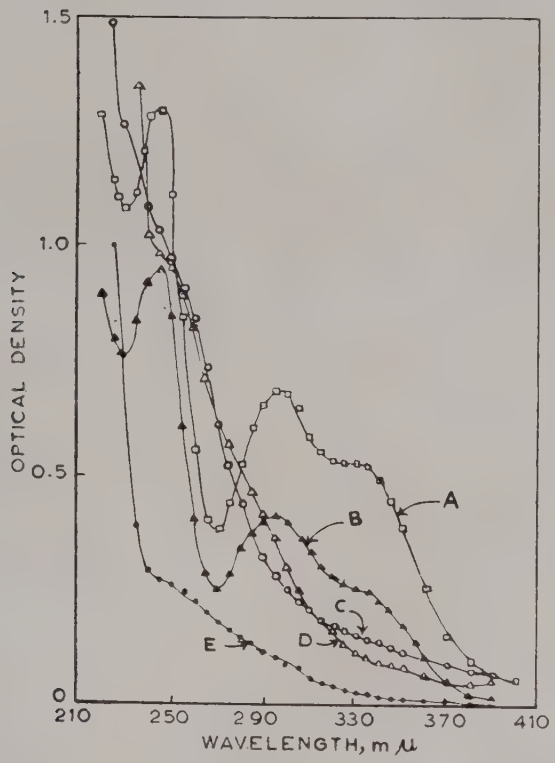

Fig. 1 — Ultraviolet absorption spectra of (A) supernatant from irradiant aqueous suspension of psoralen; (B) psoralen eluted with water from a chromatogram; (C) water eluate of intense fluorescent band (R_f 0·63); irradiation of psoralen was done in ethanol-water mixture; (D) ethanol (95 per cent) eluate of band (R_f 0·67) of irradiated psoralen and cysteine hydrochloride; and (E) ethanol (95 per cent) eluate of band (R_f 0·67) of incubated psoralen and cysteine hydrochloride (60°C. for 3 hr) mixture

Discussion

The results of the present study would indicate that the irradiation of psoralen with ultraviolet light in the presence or absence of cysteine or other sulphydryl compounds results in the production of fluorescent products other than the parent compound. These observations are similar to those of Fowlks[11] who identified a dimer and furocoumaric acid as the irradiation products of psoralen. The irradiation of 8-MOP has been reported by Lerner *et al.*[12] to bring about a loss of characteristic peaks of the absorption spectrum and a generalized absorption which becomes stronger towards the shorter wavelength region. Although the absorption spectrum of the supernatant of irradiated psoralen was similar to that of psoralen, it was possible to separate the fluorescent transformation products of psoralen using the chromatographic method described above. These products, unlike psoralen, have no maxima between 220 and 390 mμ and a generalized absorption which becomes stronger towards the shorter wavelength region. It appears from the present investigation that the two products, e.g. dimer and furocoumaric acid, are not produced as revealed by a comparison of the absorption spectra. That a fluorescent compound (R_f value 0·67) having absorption spectrum similar to that obtained by irradiation of psoralen alone or with cysteine should be produced even after incubation of psoralen suspension with cysteine at *p*H 2-3, is rather interesting and would probably point towards the possible role of psoralen in the inhibition of sulphydryl groups, which are important *in vivo* determinants of tyrosinase activity[5], and hence melanin formation in the skin. Coumarins are known to inhibit sulphydryl groups and recently the action of some aromatic thiols on coumarins has also been reported[13]; hence the possibility of inactivation of sulphydryl groups by furocoumarins and ultraviolet light probably needs special attention.

The formation of newer products from psoralen as a result of ultraviolet irradiation also appears to be interesting in view of the fact that in the treatment of vitiligo, furocoumarins have been reported to be inactive by themselves unless aided by exposure to ultraviolet light. As speculated earlier[12], it may be that one of the transformed products of psoralen may be responsible for accelerating melanin formation in vitiligo patients. This together with the photo-oxidation of inhibitors as sulphydryl groups or of DOPA to melanin, or a change in redox potentials making the conditions more favourable for normal melanin formation in the presence of psoralen and ultraviolet light, may be some of the mechanisms involved in the action of furocoumarins.

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Studies on *Idli* Fermentation: Part II—Relative Participation of Black Gram Flour & Rice Semolina in the Fermentation

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The effect of varying the concentrations of black gram flour and rice semolina on fermentation and *idli* quality has been studied. Assessment of individual and additive contributions of both the fermenting components has indicated that the fermentation is mediated and influenced by both black gram flour and rice semolina. Though its autofermenting power is poor, rice semolina contributes microorganisms and also acts as a substrate. Black gram flour plays a major role in the fermentation, both as a good source of microorganisms and also as a vigorous fermenting substrate. Microorganisms developed during presoaking of black gram flour had high autofermenting ability and also more potentialities to act on rice semolina.

THE results of earlier studies[1] indicated that the major component contributing to the fermentation in the *idli* batter is the black gram flour, the rice semolina by itself not playing a significant part. Studies relating to the assessment of the relative participation of black gram flour and rice semolina in the fermentation are reported here.

Experimental procedure

Materials — The fermentative index of mixtures of rice semolina and black gram flour in varying proportions was first determined to fix the optimum proportions at which proper fermentation and *idlis* of acceptable organoleptic quality could be obtained. At this optimum proportion the fermenting ability of the two ingredients when suspended in water separately and in combination was determined. The effect of addition of dry bakers' yeast (DCL) and mixed lactic inoculum (in the form of lactic curd) to these ingredients was also determined.

In order to determine the effect of one component on the other, either as a carrier of microorganisms or as a substrate for the fermentation, one of the components was sterilized by autoclaving, mixed with the unsterilized ingredient and allowed to ferment. In another experiment a mixture of black gram flour and rice semolina, both sterilized, was subjected to fermentation by the yeast and lactic inoculum.

The next set of experiments was designed to study the manner in which presoaking of black gram flour in water for 6 hr prior to mixing with rice semolina aids fermentation as suggested in the earlier study[1]. A water extract from sterile black gram flour and from a paste of black gram flour autofermented for 6 hr was prepared and added to rice semolina and then fermented. The effect of adding a similar extract from rice semolina presoaked for the same period to black gram flour was also studied for comparison.

Methods — The black gram flour and parboiled rice semolina used were prepared as in the previous study. Progress of fermentation was followed by studying the increase in acidity of batter as described earlier[1]. Organoleptic quality was tested on the *idli* formed from the fermented batter steam cooked for 10 min.

Sterilization of black gram flour and rice was effected by autoclaving at 15 lb./sq. in. pressure for 1 hr. The sterilized component was mixed with sterile distilled water and then incubated for 18 hr. In some studies the non-sterilized component was quickly added to the sterilized ingredient, mixed with distilled water and then fermented. The operations in these experiments were conducted with rapidity in sterile surroundings near a flame and the beakers were tightly covered immediately to prevent access to organisms from external sources.

For the inoculum studies the black gram flour (120 g.) was sterilized by autoclaving at 15 lb./sq. in. pressure for 1 hr on two successive days, extracted with sterile water (800 ml.), centrifuged twice at 2000 r.p.m. and the centrifugate again autoclaved. Black gram flour or rice semolina (120 g.) was prefermented with 400 ml. of distilled water for 5·5 hr

at 30°C. and shaken in a mechanical shaker for 30 min. after adding 400 ml. more of distilled water. The slurry was then centrifuged twice at 2000 r.p.m. and the clear extract was used. For each study 55 ml. centrifugate representing the microbial inoculum and the solubles that were extracted from 8·3 g. of black gram flour or 16·6 g. of rice semolina taken.

Results and discussion

The results reported are the averages of three independent experiments. Though variations in the actual values were sometimes observed with materials prepared at different times, reproducibility of the pattern of results was generally good.

Proportions of rice semolina and black gram flour — Increment in the black gram flour component of the fermenting mixture caused increases in the volume of the batter and total acidity (Table 1). If the proportion of black gram flour was less than 25 per cent, the steam-cooked *idlis* were hard and unacceptable organoleptically. If it was more than 50 per cent, the *idlis* tended to be sticky and were, hence, unacceptable. Best results were obtained when the proportion of rice semolina to black gram flour was 2 : 1. *Idlis* obtained from 100 per cent black gram flour were very sticky even though the batter registered a maximum increase of the gas held by the fermenting medium. Such a batter was so soft that during steaming it collapsed and a sticky *idli* was obtained.

Individual and additive contribution of black gram flour and rice semolina to fermentation — The results presented in Table 2 show that although the major contribution to acid and gas production was by the black gram component, acidity and gas production were more when black gram flour and rice semolina were fermented together than the sum total of their individual contributions. Similar results were observed even when the rice semolina was added to a prefermented black gram flour paste as in the ' flour presoaking ' method. Hence in both the ' composite mix ' method and the ' flour presoaking ' method, the fermentation appears to be mediated and influenced mutually by the fermenting components. The results further indicate that while the addition of lactic inoculum from curd increased the acidity, the addition of dry bakers' yeast increased the gas production when the lactic inoculum was also present. Sodium chloride in the doses used had no effect on either.

Influence of one component on the other during fermentation — In the results of studies reported in Table 3, all the constituents, excepting the one whose effect was to be studied, were maintained under sterile conditions by destroying the microbial load on them by autoclaving. These sterile components could act only as substrates. The results show that the organisms from rice semolina could act on sterile black gram flour as substrate and could contribute considerably to the acidity (15·2 ml.). Similarly the organisms from black gram flour acting on sterile rice semolina contributed 7·2 ml. towards acidity. When autofermented, black gram flour contributed a major share of the total acid produced (33·7 ml.). In the presence of sterile rice semolina, the black gram flour registered the maximum increase in the batter volume. The microbes in the distilled water alone could

TABLE 1 — EFFECT OF DIFFERENT PROPORTIONS OF RICE SEMOLINA AND BLACK GRAM FLOUR ON FERMENTATION AND *IDLI* QUALITY

(*Incubation temp.*, 30°C.; *incubation period*, 18 *hr*; *in all studies* 2·7 *g. of sodium chloride*, 0·9 *ml. of* 1 : 10 *diluted curds and* 1 *ml. containing* 14 *mg. of DCL yeast were added*)

Rice semolina *g.*	Black gram flour *g.*	Water *ml.*	Increase in vol. of batter *ml.*	Acidity* *ml.*	*Idli* quality
90·0	0·0	198	0	9·4	H-NA
67·5	22·5	198	85	30·2	H-NA
60·0	30·0	198	100	33·1	S-A
45·0	45·0	198	135	42·5	S-NA
30·0	60·0	198	195	52·0	ST-NA
22·5	67·5	198	205	56·0	ST-NA
0·0	90·0	198	220	61·5	ST-NA

H, hard; S, soft; ST, sticky; NA, not acceptable; and A, acceptable.

*50 g. fermenting medium in 100 ml. water was titrated against 0·1*N* sodium hydroxide.

TABLE 2 — RELATIVE PARTICIPATION OF RICE SEMOLINA AND BLACK GRAM FLOUR IN FERMENTATION

(*Incubation temp.*, 30°C.; *incubation period*, 18 *hr*)

Sl No.	Fermenting medium	Batter vol., *ml.*		Acidity (0·1*N* NaOH) *ml.*	
		Initial	Increase	Initial	Increase
1	B+W*	32	90	10·6	18·2
2	R+W*	38	0	1·5	8·3
3	B+W	59	87	10·6	35·0
4	R+W	65	0	1·5	7·9
5	B+R+W	72	196	13·0	47·8
6	B+R+W+S	72	181	13·0	46·6
7	B+R+W+S+C	72	61	13·0	76·3
8	B+R+W+S+Y	72	183	13·0	45·5
9†	B+R+W+S+Y+C	72	127	13·0	55·4
10‡	B+W* and after 6 hr R+W*+S	72	63	13·0	60·0

B, black gram flour (8·3 g.); R, rice semolina (16·6 g.); W, distilled water (55 ml.); S, sodium chloride (0·7 g.); C, 1 ml. of 1 : 40 diluted curd; and Y, 4 mg. of DCL yeast.
W*, distilled water added, 27·5 ml.
†Represents conditions for ' composite mix ' method.
‡Represents conditions for ' flour presoaking ' method.

TABLE 3 — INDIVIDUAL CONTRIBUTION OF FERMENTING INGREDIENTS TO ACID PRODUCTION UNDER ASEPTIC CONDITIONS

(*Incubation temp.*, 30°C.; *incubation period*, 18 *hr*)

Sl No.	Details of medium*	Increase in vol.† *ml.*	Titre value‡ (less blank) *ml.*	Remarks
1	**B**+**W**	0	1·10	Organisms from R acting on **B** contribute 15·2 ml. acidity
2	**B**+**W**+R	3	24·80	
3	**W**+R	0	8·50	
4	**R**+**W**	0	1·50	Organisms from B acting on **R** contribute 7·2 ml. acidity
5	**R**+**W**+B	203	42·40	
6	**W**+B	96	33·70	
7	**W**+B+R	211	46·80	—
8	**B**+**R**+**W**	0	3·30	Organisms from distilled water contribute 4·1 ml. acidity
9	**B**+**R**+W	0	7·40	
10	**B**+**R**+W+C	0	21·05	Organisms from curd contribute 13·65 ml. acidity
11	**B**+**R**+W+Y+C	13	17·50	—

*B, black gram flour (8·3 g.); R, rice flour (16·6 g.); W, distilled water (55 ml.); C, 1 ml. of 1 : 40 diluted curd inoculum; Y, 4 mg. of DCL yeast; and **B**, **R** and **W**, autoclaved materials. Distilled water was autoclaved separately and added where necessary.

†Initial vol.: 72 ml. in Sl Nos. 2, 5, 7, 8, 9, 10 and 11.

‡Zero hour blank titre values (acid produced expressed in ml. of 0·1*N* NaOH): **B**, 11·9 ml.; **R**, 1·3 ml.; **BR**, 13·85 ml.; B, 10·6 ml.; R, 1·5 ml.; and BR, 13·0 ml.

contribute only slightly to the fermentation. These experiments clearly showed that both rice semolina and black gram flour could act in the dual capacity of providing the microorganisms and also acting as substrates. In the previous study, rice semolina soaked in water for 8 hr was reported to register an increase in the total bacterial load[1] and the present observations confirm the view that though its autofermenting power was low, rice semolina could donate organisms to the fermentation and also act as a substrate. Though the individual contributions have been assessed in this study, the sum total effect of all the constituents in this synergistic fermentation when the organisms from all the materials have a chance to act may be slightly different, but likely to follow a general pattern indicated by these results. These findings represent trends of fermentation mechanism operative in the ' composite mix ' method.

Inoculum studies — Mention has been made in the earlier communication[1] that by presoaking the black gram flour for 6 hr in water and then adding the rice semolina and water, the addition of an external inoculum consisting of curd and dry bakers' yeast to control the fermentation could be avoided. The inoculum studies reported in Table 4 were planned towards understanding the changes during presoaking and its effects.

The extract of the black gram flour presoaked for 6 hr contained the organisms as well as soluble fermentable substrate, and could undergo fermentation (20·2 ml.), while the extract from sterile black gram flour could not ferment by itself due to lack of organisms. Although rice fermented very little by itself, the addition of inoculum from presoaked black gram flour caused good fermentation (23·1 ml.). If both the ingredients were together from the start, organisms from black gram flour acting on rice as substrate could contribute only 7·2 ml. (Table 3). The inoculum from rice semolina presoaked for a similar period produced negligible acidity when incubated by itself. When this inoculum was added to black gram flour, some fermentation was recorded (12·7 ml.). The results show that the organisms developed during presoaking of black gram flour for 6 hr had, in addition to the high autofermenting ability, more potentialities to act on rice semolina.

The results of all these studies point to the significant role which black gram flour plays in the fermentation, both as a source of the microorganisms and also as a vigorous fermenting substrate. Rice, which forms two-thirds of the mix, plays a relatively secondary role in the fermentation, though microorganisms from the rice were shown to act on the black gram flour in causing fermentation to a minor extent.

TABLE 4 — FERMENTING CAPACITY OF INOCULUM FROM PRESOAKED BLACK GRAM FLOUR OR RICE SEMOLINA

(*Incubation temp.*, 30°C.; *fermentation period*, 18 *hr*)

Details of inoculum*	Acidity (*ml.* 0·1*N NaOH*) contributed by			c−(a+b) *ml.*
	Inoculum (a) *ml.*	Fermenting ingredient (b) *ml.*	Inoculum mixed with fermenting ingredient (c) *ml.*	
Inoculum from sterile black gram flour	5·6	4·2R	15·0	5·2†
Inoculum from presoaked black gram flour	28·2	4·2R	55·5	23·1‡
Inoculum from presoaked rice semolina	3·3	35·2B	51·2	12·7§

B, black gram flour (8·3 g.); and R, rice semolina (16·6 g.).

*55 ml. of centrifugate were used as inoculum; at the time of adding the black gram flour or rice semolina, the values for acidity for the three inocula were 5·5, 8·0 and 1·2 ml. of 0·1*N* NaOH respectively.

†Acidity due to organisms from rice semolina acting on soluble substrate from sterile black gram flour.

‡Acidity due to organisms from presoaked black gram flour acting on rice semolina as substrate.

§Acidity due to organisms from presoaked rice semolina acting on black gram flour as substrate.

Rice is used in the form of a coarse semolina having poor extractability which could be one of the reasons for the low fermentability of rice. Even though black gram flour ferments vigorously and holds the gas formed in the batter, by itself it does not make good *idlis*. The structure of the batter rich in black gram was so soft that it collapsed completely when steamed and made the *idlis* sticky and seemed to require the rice component to offer the necessary support to the batter so that the space vacated by escaping gases during steaming would remain intact and present the familiar honeycomb structure characteristic of *idli*.

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Pharmacognostic Study of the Stem Bark of *Soymida febrifuga* A. Juss.

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The macroscopic and microscopic characters of the stem bark of *Soymida febrifuga* A. Juss. are described. Numerous corky warts on the outer surface of the stem bark are a distinguishing character. Cells with deep coloured cell contents are found distributed throughout the bark. The cell contents are mostly tannin as confirmed by microchemical test. Complete absence of stone cells, and the presence of prismatic, rhomboidal and clustered crystals of calcium oxalate are the chief diagnostic characters.

SOYMIDA FEBRIFUGA A. Juss. (Hindi: *Rohan* or *Rohra*) belongs to the family *Meliaceae*. The stem bark is considered in the indigenous system of medicine as an astringent, bitter, tonic and febrifuge, and is also used in general debility, intermittent fevers, diarrhoea and dysentery[1-6]. Chemical investigation of the stem bark shows the presence of a bitter substance, resin, gum, starch, tannic and gallic acids. As no detailed pharmacognostic studies have been made on this plant, the present work was taken up with a view to identifying the bark properly.

The plant is found in the forests of the Deccan from Kurnool to Mysore, and the hills of Chingleput[7]. It is a large tree with greyish green leaves, thick bluish grey or brown bitter bark and very hard, red and handsome valuable wood[7].

Materials and methods

Fresh barks collected from the Indian Botanic Garden, Sibpore, Calcutta, as well as from the crude drug dealers of Calcutta were compared macroscopically and microscopically. Sections of the fresh barks were cut in transverse, tangential-longitudinal and radial-longitudinal directions, stained with safranin and fast green, and made permanent. Powders passing through No. 60 B.S. sieve were used. A small portion of the powder was cleaned with chloral hydrate for studies of different elements present in the powdered bark. A portion of the fresh bark was macerated and examined. Sections of the fresh bark were also cut for the standard microchemical tests[8] for the study of the chemical constituents present in the stem bark.

Macroscopic characters (Plate I)—The stem bark is about 5-15 mm. thick and occurs in slightly curved quills. Externally, it is of a rusty grey or

Plate I — Stem bark of *S. febrifuga* × 1

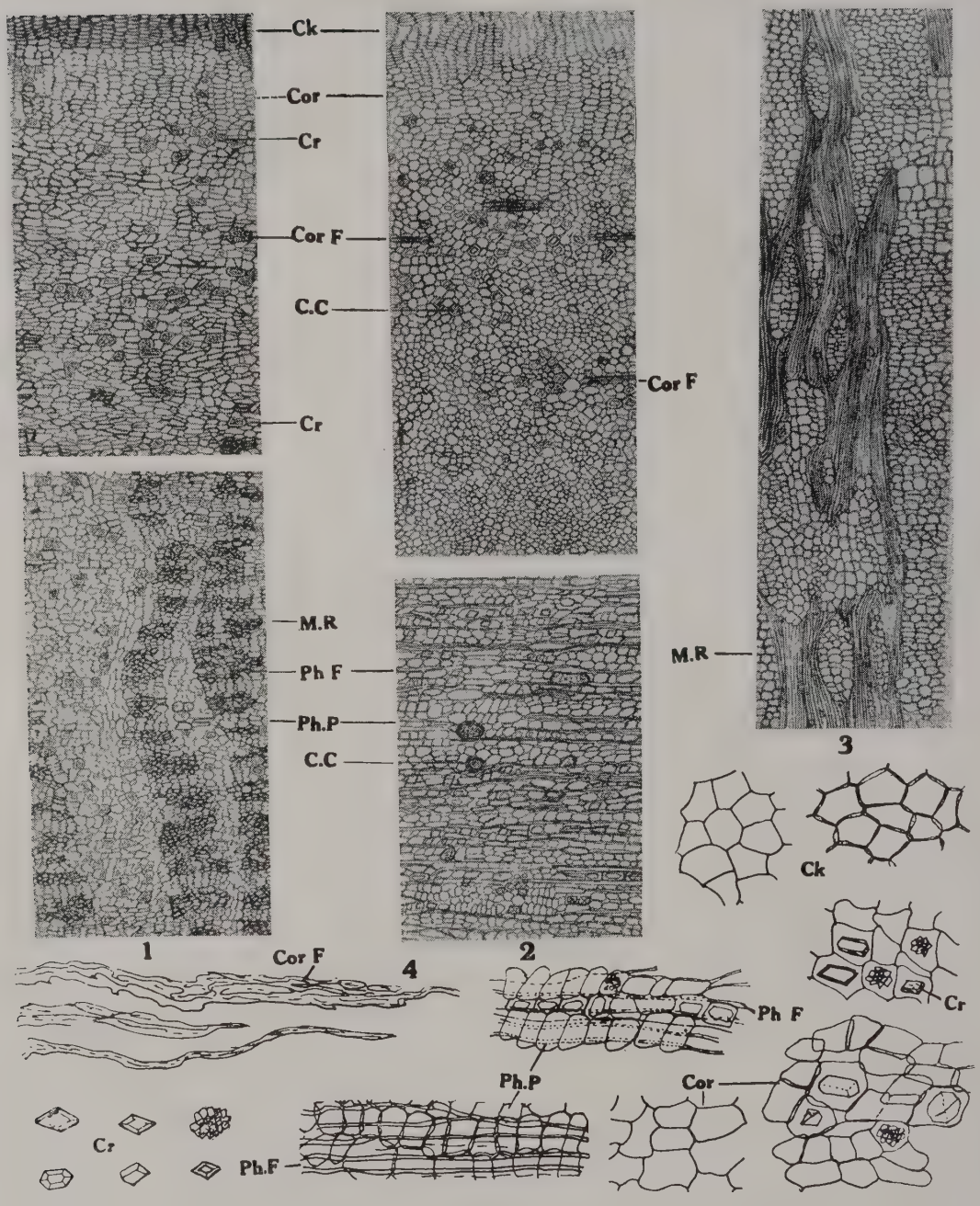

Plate II—Microscopic characters of stem bark [Fig. 1: T.s. of bark; upper half shows details of periderm region and lower half shows details of phloem region × 250. Fig. 2: R-l section of bark; upper half shows details of periderm region and lower half shows details of phloem region × 250. Fig. 3: T-l section of bark × 250. Fig. 4: Diagnostic elements of the powdered bark × 150. *C.C*, cells with tannin; *Ck*, cork cells; *Cor*, cortical parenchyma cells; *Cor.F*, cortical fibres; *Cr*, crystals; *M.R*, medullary ray cells; *Ph.F*, phloem fibres; *Ph.P*, phloem parenchyma cells]

brown colour with a rough surface and longitudinal furrows. Numerous small corky warts are found in the outer surface. The inner side and edges of the quills are of a bright reddish brown colour. Fracture very firm, dense and fibrous.

Microscopic characters — In transverse section, the periderm consists of 10-15 layers of thick-walled cork cells, the inner walls of which are lignified. The cork cells are brick-shaped and tangentially elongated. The cork cells measure T = 12-**18**-23 μ; R = 25-**35**-46 μ and L = 12-**23**-35 μ (Plate I, Figs. 1 and 2); T, R and L denote measurements taken in tangential, radial and longitudinal directions. The phellogen or cork cambium is 1-3 layered and similar to those of phellem but are thin-walled. The phelloderm or secondary cortex, which occupies about 30 per cent of the total thickness of the bark, consists of cortical parenchyma and cortical fibres (Plate II, Figs. 1 and 2). The cortical parenchyma cells are thin-walled, parenchymatous with different sizes and shapes. They are generally tangentially elongated. Prismatic and clusters of calcium oxalate crystals are generally found in this region. Starch grains are found in small numbers in the upper part of the cortical cells. Unlike the general cortical cells some cells are found with deep coloured cell contents (Plate II, Fig. 2). The cortical parenchyma cells give positive test for tannin and measure T = 23-**62**-96 μ; R = 16-**32**-46 μ and L = 16-**35**-53 μ. Associated with the cortical parenchyma lie the cortical fibres which are generally large with narrow lumen. The walls of the cortical fibres are wavy with pointed ends (Plate II, Fig. 4). The fibres are lignified and measure 2-5 mm. in length and 9·2-23 μ in breadth. Next to this region is the phloem which occupies about 70 per cent of the total thickness of the bark and consists of phloem parenchyma, phloem fibres, medullary rays, sieve tubes and companion cells. The phloem parenchyma and phloem fibres are arranged alternately (Plate II, Figs. 1 and 2). The phloem parenchyma are traversed by medullary rays. The phloem parenchyma are thin-walled cells which are smaller in size in comparison to the cortical parenchyma. They do not contain starch grains. Phloem parenchyma cells give positive test for tannin as confirmed by microchemical test. The phloem parenchyma also contain crystals of calcium oxalate which are either solitary, clustered or rhomboidal. These cells measure T = 14-**37**-52 μ; R = 16-**21**-28 μ and L = 14-**23**-36 μ. The phloem fibres are also thick and wavy. The fibres lie either scattered in small groups or in bands and are generally lignified as confirmed by microchemical test. The phloem fibres measure 0·5-2·5 mm. in length and 18·4-20·9 μ in breadth. The medullary rays are radially elongated thin-walled cells which may be 2-6 layered in width and contain tannin (Plate II, Figs. 1 and 3). The cells measure T = 32-**58**-71 μ; R = 18-**35**-51 μ and L = 18-**37**-58 μ. In the secondary phloem region large cells packed with reddish brown cell contents are found which measure T = 35-**53**-104 μ; R = 23-**46**-69 μ and L = 23-**60**-92 μ (Plate IIB, Fig. 2).

Microchemical examination of bark sections — Treatment of the bark sections with standard chemical test reagents revealed the presence of starch grains in cork and cortical cells, tannins in phloem parenchyma, lignin in the inner walls of cork cells, and cortical and phloem fibres, crystals of calcium oxalate in cortical and phloem parenchyma cells, and gums and resins in the secondary phloem region. Mucilage, cellulose and alkaloids were not detected in any of the sections.

Powder (Plate II, Fig. 4)— Brick-red in colour with fragrant odour and almost without any marked taste. The powder when examined under the microscope is characterized by the presence of isolated groups of thick-walled cork cells, cortical parenchymatous cells, with or without prismatic, clustered and rhomboidal crystals of calcium oxalate, isolated or scattered phloem fibres, scattered phloem parenchyma and prismatic, clustered and rhomboidal crystals of calcium oxalate.

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Pharmacognosy of the Root of *Agrimonia eupatoria* Linn.

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The macroscopic and microscopic characters of the root of *A. eupatoria* Linn., which is reputed to be an astringent and tonic, are described. The diagnostic character of the root is the presence of polyderm, a special type of protective tissue.

AGRIMONIA EUPATORIA Linn. Agrimony, belonging to the family *Rosaceae*, is a hardy perennial herb with erect stems and alternate oddly pinnate compound leaves. It grows 2-3 ft high, in small clumps, from a short root-stock. It occurs in the temperate Himalayas from Murree and Kashmir (altitude 3000 to 10,000 ft) to Sikkim (altitude 7000 to 10,000 ft) and in the Khasia (altitude 4000 to 6000 ft) and Mishmi Hills[1]. It is cultivated in many herb gardens to make a tonic tea from its leaves[2] and the root is used as an astringent and tonic[3,4]. It is considered to be a diuretic and its decoction is reported to be useful in the treatment of cough, simple diarrhoea and relaxed bowels. In India, the drug has not so far been commercially exploited. In Europe, the plants are collected from wild sources and marketed chiefly in Germany, Italy, Czechoslovakia and Hungary[5]. The root has a somewhat bitter spicy taste but no perceptible odour.

Agrimonia eupatoria Linn. is often adulterated with *A. odorata* (Gouan) Mill. in Europe and with *A. striata* and *A. shaveolens* in America. Many of the subspecies are, however, not clearly distinguished from one another and noteworthy local, regional and climatic differences exist in the two important forms, *A. eupatoria* and *A. odorata*[6]. The main feature which differentiates the two species is the presence of a conical calyx tube with erect bristles which is furrowed only up to halfway in the former and a campanulate deeply furrowed calyx tube with downwardly bent bristles in the latter[5]. According to Hooker these species do not occur in India but *A. pilosa* Ladeb, which is similar to the Indian form of *A. eupatoria* described here, is reported. The *A. pilosa* plant, however, is more glabrous and the calyx tube is almost glabrous with erect bristles[1].

Materials and methods

The roots were obtained from fresh plants supplied through the courtesy of the Regional Botanist, Botanical Survey of India, Eastern Circle, Shillong, from the Khasia Hills, Assam. The identity of the material was confirmed by comparison with authentic material available in the herbarium of the Botany Division of this Institute. Roots were fixed for microtome sectioning in F.A.A. Dehydration was done by the tertiary butyl alcohol method. For maceration of root material Jeffrey's method[7] was employed and this gave very satisfactory results. A few representative roots and rootlets were dried and later on powdered. The powder was taken after sifting through a No. 80 B.S. sieve. Hand sections of the fresh and dry material were cut for microchemical tests and microtome sections were cut at 15 μ for study of the anatomical structures. The figures were drawn with the help of a prism type camera lucida.

Macroscopical characters

Individual roots are cylindrical, unbranched and tapering and vary from 3·0 to 12·0 cm. in length and from a fraction of a mm. up to 4·0 mm. in diam. The roots bear numerous lateral rootlets (Plate I, Fig. 1, *rl*). The outer surface of the root is dark brown in colour and is smooth. A smoothed transversely cut surface reveals a core of a yellowish wood which occupies from one-half of the diameter of the entire root in the younger roots to one-third of the root diameter in the mature and older roots. The remaining one-half to two-thirds of the diameter of the root comprises mostly the yellowish brown periderm (Plate I, Fig. 2, *pd*). In the dried roots, fracture is short and starchy.

Microscopical characters

On the outside lies the polyderm (Plate I, Figs. 2-5, *p*), a special type of protective tissue reported to occur in the roots and underground parts of plants belonging to the families *Hypericaceae, Myrtaceae, Onagraceae* and *Rosaceae*[8-10]. The polyderm is delimited from the outermost layer of the secondary cortex by a single layer of meristematic rectangular thin-walled cells which constitute the phellogen

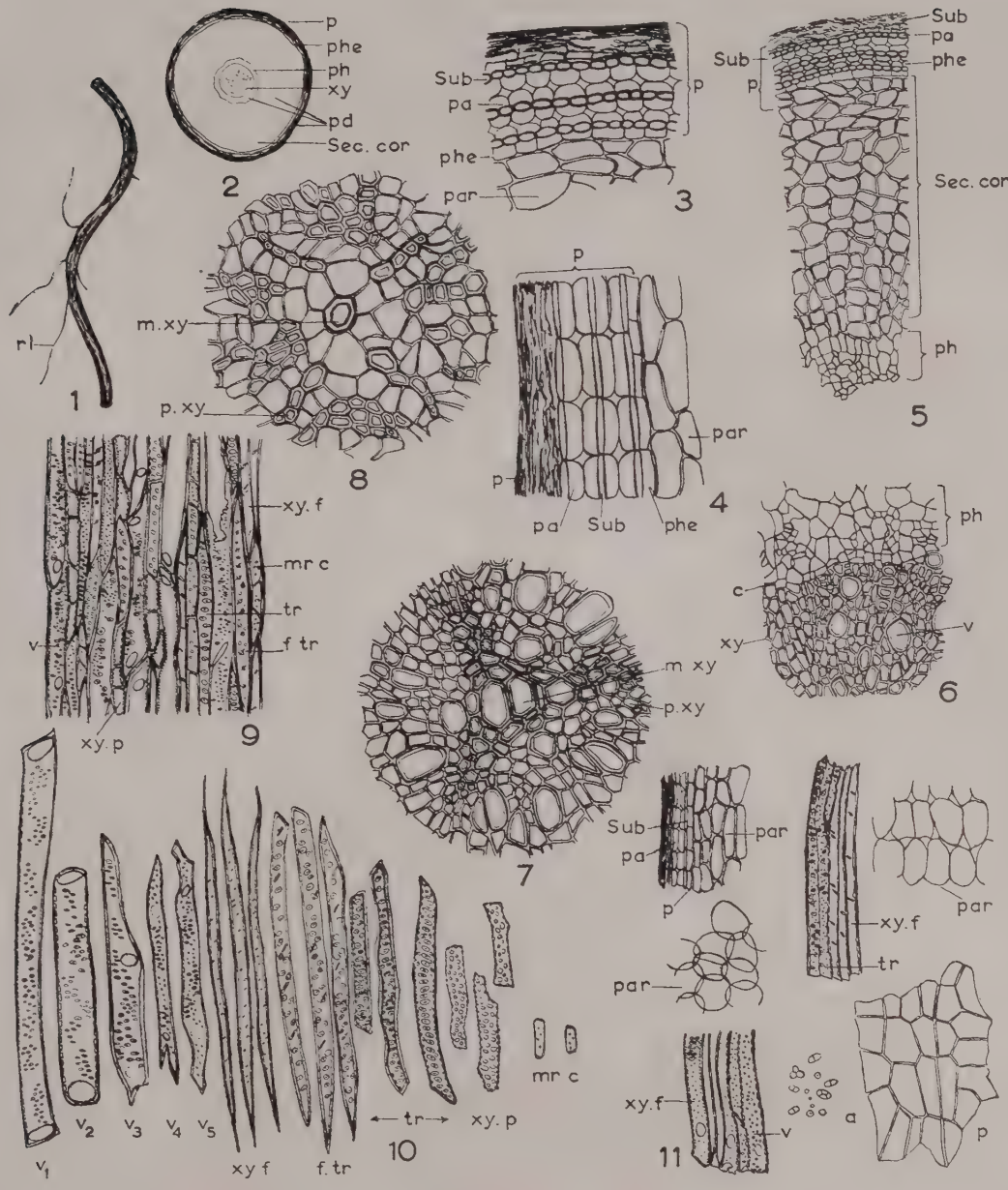

Plate I — *Agrimonia eupatoria* Linn. root [Fig. 1: Macroscopic appearance of the root × 0·6. Fig. 2: Diagrammatic t.s. of the root × 12. Fig. 3: Part of a t.s. through the polyderm region of the root × 156. Fig. 4: Part of a l.s. through the polyderm region of the root × 156. Fig. 5: Part of a t.s. through the polyderm and secondary cortex regions of the root × 78. Fig. 6: Part of t.s. through the secondary phloem and xylem portions of the root × 156. Fig. 7: T.s. through the central portion of a young root × 216. Fig. 8: T.s. through the central portion of an old root × 216. Fig. 9: Tangential l.s. through a portion of the wood × 108. Fig. 10: Elements for chromic-nitric acid maceration of the wood portion of the root × 108. Fig. 11: Powdered root × 156. *p*, polyderm; *phe*, phellogen; *par*, parenchyma; *ph*, phloem; *xy*, xylem; *pd*, periderm; *xy.f*, wood fibre; *xy.p*, xylem parenchyma; *m.r*, medullary ray; *mr.c*, medullary ray cell; *v*, vessel; *tr*, tracheid; *p.xy*, primary xylem; *a*, starch grains; *m.xy*, metaxylem; *sub*, suberized cells of the polyderm; *s.c*, secondary cortex; *pa*, parenchymatous cells of polyderm]

(Plate I, Figs. 3-5, *phe*). The phellogen cuts off externally layers of uniseriate oval or dumb-bell-shaped suberized cells resembling the endodermis of other dicotyledonous roots but devoid of any starch contents (Plate I, Figs. 3-5, *sub*). External to this uniseriate suberized layer are two layers of more or less rectangular, thin-walled parenchyma cells with conspicuous intercellular spaces (Plate I, Figs. 3-5, *pa*). With iodine, the contents of these cells give a positive test for starch. Next to these parenchyma cells externally is again a uniseriate layer of suberized cells followed by biseriate layer of thin-walled parenchyma cells and then again a uniseriate suberized layer. The polyderm is thus constituted by uniseriate layers of endodermoid cells and the intervening layers of more or less rectangular thin-walled cells which alternate with each other. The outermost portion of the polyderm is dark brown in colour and broken at places and is composed of dead cells. The suberized cells (Plate I, Figs. 3-5, *sub*) of the polyderm measure R = 8-**10**-16 μ; L = 51-**89**-138 μ and T = 16-**22**-32 μ. The intervening parenchyma cells of the polyderm measure R = 12-**15**-20 μ; L = 51-**71**-118 μ and T = 20-**29**-43 μ. The secondary cortex (Plate I, Figs. 2 and 5, *s.c*) is a wide zone of 18-20 layers of thin-walled oval or rounded parenchyma cells which are arranged radially at places and measure 28-**51**-75 μ in diam. These cells contain abundant starch grains. Individual starch grains are either simple, circular or oval in outline or 2-4-compound and measure 12-**14**-16 μ in diam. Internal to the secondary cortex is the secondary phloem (Plate I, Figs. 5 and 6, *ph*) which is composed of sieve tubes, companion cells, phloem parenchyma and few medullary ray cells.

The xylem (Plate I, Figs. 2 and 6, *xy*) constitutes almost one-half of the total diameter in a young root but in the older roots it occupies only up to one-third of the entire diameter of the root. The primary xylem is triarch or tetrarch (Plate I, Figs. 7 and 8, *p.xy*). The secondary xylem is composed of vessels, tracheids, xylem fibres, xylem parenchyma and medullary ray cells, all of which are lignified (Plate I, Figs. 6 and 9, *xy*). The vessels may be very long or are short, being about half the size of the long ones; they occur singly or at places in linear rows of two vessels. The vessel diameter in a transverse section varies from 15 to 30 μ. The vessels bear bordered pits on the side walls and the pitting is more between contiguous vessels and tracheids. The vessels measure 229-**329**-443 μ in length from end to end and 20-**36**-58 μ in breadth. The tracheids have bordered pits on the side walls similar to those on the vessels. These measure 122-**244**-336 μ in length and 16-**19**-28 μ in breadth. The xylem fibres are of two types — the fibre tracheids and wood fibres (Plate I, Fig. 10, *f.tr, xy.f*). The fibre tracheids are aseptate and have bordered pits on their side walls but the pit cavities are smaller than the pit cavities seen in vessels and tracheids. The wood fibres (Plate I, Fig. 10, *xy.f*) are also aseptate with bordered pits but are somewhat narrower than the fibre tracheids and the ends are more pointed. The two types of xylem fibres measure 430-**640**-860 μ in length and 8-**13**-20 μ in breadth. The xylem parenchyma cells (Plate I, Fig. 10, *xy.p*) are rectangular in outline and bear a number of rounded or oval simple pores. These measure 75-**110**-142 μ in length and 12-**16**-24 μ in breadth. The medullary ray cells (Plate I, Fig. 10, *mr.c*) are few and are quite narrow and rectangular in shape; these have slightly thickened walls, bear a few simple pits and measure 40-**53**-71 μ in length and 10-**12**-14 μ in breadth.

Powdered root

The powder of the root is brownish pink to dirty pink in colour. The salient microscopic characters are: (1) Presence of fragments of polyderm layers with the outer dead cells and the alternating layers of suberized endodermoid cells and the intervening biseriate parenchyma cells (Plate I, Fig. 11, *p, sub, pa*); (2) patches of outermost cells of polyderm (Plate I, Fig. 11, *p*); (3) abundant simple or 2-4-compound starch grains, circular or oval in outline, and measuring 12-16 μ in diameter (Plate I, Fig. 11, *a*); (4) parenchyma cells from secondary cortex (Plate I, Fig. 11, *par*); (5) fragments of lignified xylem elements, viz. vessels, tracheids, fibre tracheids, wood fibres, xylem parenchyma, etc., with their characteristic pittings; and (6) a lot of cell debris.

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Studies on 4-Arylthiosemicarbazones & Related Products: Part I—Synthesis of Some 4-Arylthiosemicarbazones as Potential Antitubercular Agents

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Thirty-four 4-arylthiosemicarbazones with typical substituents at the 1- and 4-positions have been synthesized. These have been screened for their inhibitory effect *in vitro* on *Mycobacterium tuberculosis*. The aldehyde moiety in these compounds influences the activity more than substituents at the 4-aryl group. 1-Fural-4-*p*-tolylthiosemicarbazone has been found to possess maximum activity.

THE advent of Tibione (*p*-acetaminobenzalthiosemicarbazone) as a reputed, clinically effective tuberculostat[1-7] brought to the fore thiosemicarbazones as a group of antitubercular agents. Extensive investigations have, therefore, been carried out on the tuberculostatic activity of closely related thiosemicarbazones with the hope of arriving at atoxic substances of enhanced activity[8-15]. Tisler's work[16,17] has shown that 4-arylthiosemicarbazones also possess promising antitubercular activity *in vitro*. The results of other workers[18,19] corroborate these findings. This communication describes the synthesis of a series of 4-arylthiosemicarbazones and the results of their screening, *in vitro*, for antitubercular activity.

Thirty-four thiosemicarbazones have now been synthesized by condensing 4-phenyl, 4-*p*-tolyl and 4-*p*-chlorophenyl thiosemicarbazides with various aromatic aldehydes possessing typical substituents and one heterocyclic aldehyde. The yields, physical characteristics and analytical data of these compounds are given in Table 1.

When tested *in vitro* upon *Mycobacterium tuberculosis*, compounds 1, 7, 11, 13, 14, 19, 29, 30 and 32 inhibited the growth of bacteria at a dilution of 10 γ/ml., compounds 3, 8, 17, 22 and 26 at 4 γ/ml. and compound 25 at 2 γ/ml. The rest of the compounds were not active even at a concentration of 10 γ/ml. Dihydrostreptomycin sulphate, which was used as a standard, had inhibitory effect at 0·2 γ/ml.

Some broad features regarding the structure-tuberculostatic activity relationship are evident from these results. The aldehyde moiety of these compounds seems to influence their activity to a greater extent than the 4-aryl group. 4-Arylthiosemicarbazones derived from *p*-tolualdehyde are less active than those from benzaldehyde. Introduction of halogen atoms or a *p*-dimethylamino group renders these compounds less active. However, a nitro group appears to confer increased activity. Maximum activity is reached in 1-(2′-fural)-4-*p*-tolylthiosemicarbazone, which indicates that thiosemicarbazones from oxygen heterocyclic aldehydes may possess a favourable structure for tuberculostatic activity.

Experimental procedure

All melting points are uncorrected.

4-Phenylthiosemicarbazide[20], 4-*p*-tolylthiosemicarbazide[23] and 4-*p*-chlorophenylthiosemicarbazide[16] were prepared by treating the corresponding isothiocyanates with hydrazine hydrate.

General procedure for the preparation of 4-arylthiosemicarbazones — Equimolecular quantities of the 4-arylthiosemicarbazide and the aldehyde were refluxed in alcoholic solution on a water bath for 30 min. While in some cases the product separated out even during the course of the reaction, in others it was separated by precipitation with a suitable volume of cold water. The crude product was purified by crystallization from a suitable solvent.

Tuberculostatic activities — The inhibitory effects of these compounds were determined against the $H_{37}Rv$ strain of *M. tuberculosis* in Kirschner's medium containing Tween-80 and enriched by 10 per cent horse serum. The compounds were taken up in 70 per cent ethanol at a concentration of 1 mg./10 ml. In the case of compounds 5, 16, 22 and 32, three drops of sterile sodium hydroxide (0·01*N*) were added to effect clear solution. Controls with ethanol, and ethanol containing a trace of alkali showed that a

TABLE 1 — 4-ARYLTHIOSEMICARBAZONES

R—NH—CS—NH—N=HC—R′

Sl No.	R′	M.P. °C.	Yield %	Mol. formula	Nitrogen, % Found	Nitrogen, % Calc.
		R = PHENYL				
1	Phenyl	192[20]	90	—	—	—
2	*p*-Tolyl	180	99	$C_{15}H_{15}N_3S$	15·8	15·6
3	*m*-Hydroxyphenyl	230	98	$C_{14}H_{13}N_3OS$	16·0	15·5
4	*p*-Methoxyphenyl	180[21]	99	—	—	—
5	*p*-Chlorophenyl	198	97	$C_{14}H_{12}ClN_3S$	14·3	14·5
6	2,4-Dichlorophenyl	186	98	$C_{14}H_{11}Cl_2N_3S$	12·6	13·0
7	*o*-Nitrophenyl	201[22]	93	—	—	—
8	*m*-Nitrophenyl	194[20]	90	—	—	—
9	*p*-Dimethylaminophenyl	212*	98	$C_{16}H_{18}N_4S$	19·2	18·8
		R = *p*-TOLYL				
10	Phenyl	170[23]	94	—	—	—
11	*p*-Tolyl	184	90	$C_{16}H_{17}N_3S$	14·8	14·8
12	*m*-Hydroxyphenyl	199	98	$C_{15}H_{15}N_3OS$	15·2	14·7
13	*p*-Hydrophenyl	173[24]	96	—	—	—
14	2,4-Dihydroxyphenyl	Softens at 145, melts with decomp. at 165	94	$C_{15}H_{15}N_3O_2S$	14·5	14·0
15	4-Hydroxy-3-methoxyphenyl	182[25]	85	—	—	—
16	3,4-Methylenedioxyphenyl	203[25]	97	—	—	—
17	*p*-Methoxyphenyl	179[24]	99	—	—	—
18	*o*-Chlorophenyl	202[25]	91	—	—	—
19	*p*-Chlorophenyl	165	98	$C_{15}H_{14}ClN_3S$	14·2	13·8
20	2,4-Dichlorophenyl	223 (decomp.)	94	$C_{15}H_{13}Cl_2N_3S$	12·6	12·4
21	*p*-Bromophenyl	160[25] (decomp.)	96	—	—	—
22	*o*-Nitrophenyl	198[24]	96	—	—	—
23	*m*-Nitrophenyl	214[24]	98	—	—	—
24	*p*-Dimethylaminophenyl	195[24]	98	—	—	—
25	2-Furyl	160-1[25]	88	—	—	—
		R = *p*-CHLOROPHENYL				
26	Phenyl	201[16]	85	—	—	—
27	*p*-Tolyl	191	91	$C_{15}H_{14}ClN_3S$	13·6	13·8
28	*m*-Hydroxyphenyl	211	74	$C_{14}H_{12}ClN_3OS$	14·2	13·8
29	*p*-Methoxyphenyl	191[16]	85	—	—	—
30	*p*-Chlorophenyl	197	88	$C_{14}H_{11}Cl_2N_3S$	13·1	13·0
31	2,4-Dichlorophenyl	224 (decomp.)	91	$C_{14}H_{10}Cl_3N_3S$	11·8	11·7
32	*o*-Nitrophenyl	212	89	$C_{14}H_{11}ClN_4O_2S$	16·5	16·7
33	*m*-Nitrophenyl	216 (decomp.)	95	$C_{14}H_{11}ClN_4O_2S$	17·1	16·7
34	*p*-Dimethylaminophenyl	176	88	$C_{16}H_{17}ClN_4S$	17·4	16·9

The compounds were generally crystallized from ethyl alcohol with the exception of Sl Nos. 7 and 8 (aqueous acetone), Sl No. 9 (nitrobenzene), Sl Nos. 23 and 31 (ethyl acetate) and Sl No. 34 (benzene).

*Gheorghiu *et al.*[26] report m.p. 197°C. for this compound.

concentration up to 2·8 per cent of these solvents had no inhibitory effect on the growth of the bacteria in the media with the quantity of the inoculum employed, but partial inhibition occurred at a concentration of 7 per cent. To 4·5 ml. of the medium were added 0·5, 0·2 and 0·1 ml. of the solution of the compounds, the volumes being finally made up to 5·0 ml. A weighed quantity of a three weeks old culture of the organism from a Lowenstein-Jensen slope was lightly ground in the medium and two drops (corresponding to 0·05 mg. of the bacteria) of the suspension were added to each tube.

The minimum inhibitory concentrations were obtained by visual comparison of growth in the medium after incubating the tubes for a period of 14 days.

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Density of Bacterial Spores & Their Destruction Rate by Heat*

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Spore suspensions of five aerobes and two anaerobes have been fractionated by centrifugation into denser and lighter fractions and the spores in each fraction tested for their destruction rates by heat. Spores of *B. stearothermophilus, B. brevis* (320), *B. subtilis* (b), *B. megaterium* and *Clostridium sporogenes* (3679) in the denser fractions have been found to be more resistant to heat than the spores in lighter fractions. No marked difference has been observed between the rates of destruction of spores of *B. cereus, Cl. sporogenes* (112) and *Cl. bifermentans* from denser and lighter fractions. The shape of destruction rate curves for spores in denser and lighter fractions does not show any change.

THERE is much evidence to indicate that the death rate of bacteria is logarithmic in nature, i.e. the normal rate of destruction of bacteria is constant. According to Hinshelwood[1], the exponential curve of the number of survivors against time can be explained if the chances of death are increased with the time of exposure and a special distribution of natural resistance in the initial population is assumed. This initial variation in the resistance of spores may depend upon their density which is suggested by the different rates at which spores settle down when spun in a centrifuge.

The first observation on the greater resistance of denser spores of *Clostridium sporogenes* (P.A. 3679) was made by Yesair and Cameron[2]. Similar observations were made by Sugiyama[3] with *Cl. botulinum* spores. In these studies, spores were used directly after fractionation from the growth medium and no attempt was made to wash the spores. The denser fractions thus obtained are likely to contain particulate debris of organic matter from the medium which could contribute to the increased heat resistance of the spores[4]. Also, these workers determined the end point of destruction with different fractions of a single suspension. This gave no idea as to whether the greater resistance shown by the denser fraction of the spores was due to a few spores only or the entire fraction was composed of spores having greater heat resistance.

If a particular spore suspension contained a mixture of spores differing in density and hence heat resistance, it was thought that it may be possible to fractionate the spores into more uniform suspensions and alter the shape of the destruction rate curves by mixing them in different proportions.

*The investigations reported here were carried out as part of postgraduate studies at the Division of Food Preservation, C.S.I.R.O., Homebush (N.S.W.), Australia, during 1953-55.

Materials and methods

Organisms — Five aerobes, viz. *Bacillus stearothermophilus*, *B. brevis* (320), *B. subtilis* (b), *B. megaterium* and *B. cereus*, and two anaerobes, viz. *Clostridium sporogenes*, strains 112 and P.A. 3679, and *Cl. bifermentans*, were used. The spores were prepared as described earlier[5].

Fractionation of spores — The spores were harvested as usual and washed with glass distilled water. Their suspensions in water were heated for 10 min. at 80°C. to destroy the vegetative cells. The heat-treated suspensions in water were spun for 2-5 min. at 1500 r.p.m. depending on the particular organism. The fractions of spores settling down were resuspended and again spun at the above speed for the same time; this process was repeated thrice to collect spores of a uniform density. The fraction (most dense) collected was suspended in 0·55*M* phosphate buffer (fraction I). The supernatants from these lots were pooled together and centrifuged for 5-8 min. at 1500 r.p.m. The spore deposits after washing three times were suspended in phosphate buffer (fraction II). The supernatants from fraction II were spun for 20-30 min. at 3500 r.p.m. depending upon the time the spores of a particular organism took to settle down completely. This fraction was also washed as above and comprised of lightest spores (fraction III). The supernatants left from these fractions were found to be quite clear and free from spores. Each fraction of a suspension was diluted approximately to the same level of initial counts before subjecting the spores to heat resistance test.

Heat resistance — The method used for studying the rate of destruction of spores in different spore suspensions was essentially based on Bigelow and Esty's 'thermal death time tube' method[6]. Spores from the heaviest and the lightest fractions (1·5 ml. in phosphate buffer) were filled in $3\frac{1}{2} \times \frac{3}{8}$ in. pyrex ampoules and heated in a water bath. The heated spores were enumerated on different media[5]. Thedestruction rate curves were plotted on semi-logarithmic coordinates, the time being plotted on a linear scale and the number of survivors on the log scale. Decimal reduction times (D values), i.e. the time interval indicated by the destruction curve passing over one log cycle, were read from the time axis.

Results and discussion

The results obtained indicate that spores of *B. stearothermophilus*, *B. brevis* (320), *Cl. sporogenes* (3679), *B. subtilis* (b) and *B. megaterium* in denser fractions, as indicated by the thermal death times (TDT) for a million-fold (10^{-6}) reduction, were slightly more resistant than the spores in the lighter fractions (Table 1). The extent of difference in TDT values for each fraction was based on the nature of the organism. These differences were not as large as those observed by Yesair and Cameron[2]. Spores of *Cl. sporogenes* in denser fractions were found by Yesair and Cameron[2] to be twice as resistant as the spores in lighter fractions; this difference was much less marked in the present studies. There was no evidence of any difference in the heat resistance of spores of *Cl. sporogenes* (112), *Cl. bifermentans* and *B. cereus* in either of the fractions.

The average ratios of decimal reduction times of spores in heavier (FI) and lighter (FIII) fractions were not much greater than unity in the case of

TABLE 1 — THERMAL DEATH TIMES FOR DIFFERENT SPORE FRACTIONS

Organism	Heating temp. °C.	TDT, *min.*	
		Fraction I	Fraction III
B. stearothermophilus	115	130	115
B. brevis (320)	110	182	165
Cl. sporogenes (3679)	110	64	50
B. subtilis (b)	95	80	65
B. megaterium	90	39	34
B. cereus	90	73	73
Cl. sporogenes (112)	100	96	96
Cl. bifermentans	95	90	90

TABLE 2 — DECIMAL REDUCTION TIMES (D VAL.) OF SPORE FRACTIONS

Organism	Heating temp. °C.	Time (*min.*) for D val.						$\frac{\text{D val.}_{FI}}{\text{D val.}_{FIII}}$
		Fraction I			Fraction III			
Spores/ml.:		10^4-10^3	10^3-10^2	10^2-10^1	10^4-10^3	10^3-10^2	10^2-10^1	
B. stearothermophilus	115	27·8	21·6	18·0	22·8	20·6	19·0	1·08
B. brevis (320)	110	31·0	31·0	31·0	28·0	29·0	29·0	1·08
Cl. sporogenes (3679)	110	12·5	9·8	8·5	10·4	7·5	6·5	1·03
B. subtilis (b)	95	7·8	6·3	5·5	6·5	5·9	5·5	1·09
B. megaterium	90	5·25	5·5	5·5	5·0	4·8	5·0	1·10
B. cereus	90	10·8	10·4	10·6	10·8	10·4	10·6	1·00
Cl. sporogenes (112)	100	13·0	13·0	13·0	13·0	13·0	13·0	1·00
Cl. bifermentans	95	14·5	13·8	12·5	14·5	13·75	12·5	1·00

spores of *B. brevis* (320), *B. subtilis* (b), *B. megaterium*, *Cl. sporogenes* (3679) and *B. stearothermophilus* (Table 2). This indicates that spores of these organisms in lighter fractions do not die at a much faster rate than spores in heavier fractions.

The decimal reduction times for spores in fraction I as compared to spores in fraction III are more marked when the reduction from 10^4 to 10^3 counts/ml. is considered. As heating proceeds and the percentage of survivors is reduced, the decimal reduction times for the two fractions come closer, and for a reduction of 10^2-10^1 counts/ml., there is virtually no difference in decimal reduction times between the two fractions. This shows that spores in denser fractions are either not very homogeneous in resistance and the fractions contain spores of high as well as low resistance, or that death was due to some sort of lethal mutation. The equalization of D values for both fractions towards the end of the destruction rate curve shows that spores in lighter fractions too contained spores of heat resistance as high as those in heavier fractions. Also, the shape of the destruction rate curves for the denser and lighter spore fractions of the bacteria does not show any marked change.

If the density of spores had any influence on the resistance of the spores, wide variations in the nature of destruction curves of either fraction could be expected. Also, if the hypothesis that the distribution of graded resistance in a spore suspension is a function controlling the shape of the destruction curves, factors other than density may also be involved. If the hypothesis that there is a difference in the density of spores in a suspension is correct, some reasons may be adduced to explain this difference. Whether this difference in the density of spores is due to variation in the size of the spores in each suspension or due to some basic difference in the internal condition of the spores cannot be judged from the present studies.

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Short Communications

Effect of Temperature on Gastric Secretion

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The effect of varying environmental temperature on histamine induced gastric secretion in male, healthy dogs with gastrostomy has been investigated by placing them in an air-cooled room maintained at a temperature 4°C. lower than the surrounding atmosphere, or in a room heated with radiators to a temperature 8°C. higher than its surroundings respectively; the effect of raising the body temperature by 1°C. produced by the administration of TAB vaccine has also been studied. In all cases a fall has been observed in the volume, and free and total acidity of the gastric secretion.

Bisgard and Nye[1] found that slight variations in environmental temperature do not influence gastric secretion, but large variations depress it. Diminution of gastric secretion with lowering of body temperature has been reported by some workers[2-4], while others[5,6] have reported diminished gastric secretion with rise in body temperature. In view of these conflicting reports it was of interest to study the effect of varying environmental and body temperatures on gastric secretion.

Healthy male dogs, after gastrostomy according to Ssbanajew-Franck (stomach cone transfer) technique[7], were employed in these studies. Histamine acid phosphate (BDH) was used to stimulate gastric secretion. By trials it was found that 0·06 mg./kg. of histamine induced optimum gastric secretion. Histamine induced gastric secretion in the animals for half an hour, before and after treatment, was collected and measured. The free and total acidity of the secretion was estimated by titrating it against 0·01*N* NaOH solution.

Exposure to cold atmosphere — Five dogs were kept in an air-cooled room at a temperature 4°C. lower than the prevailing external temperature for 4 hr.

Exposure to warm atmosphere — Five dogs were kept in a room heated by radiators. Within 30 min. the temperature around the animal was 8°C. higher than the initial temperature and was maintained at that level. The rectal temperature of the dog during the experiment did not vary.

Raising of body temperature — TAB vaccine (BCPW) was used as the pyrogenic agent. Two ml. of the vaccine produced 1°C. rise in rectal temperature. Eight such experiments were performed.

The results given in Table 1 show that when the dogs are exposed to cold or warm environment, not sufficient to cause a change in the body temperature, there is a decrease in the volume of gastric secretion and also its free and total acidity. This may be due to the change in environmental temperature acting as a stress[8]. The results are statistically significant. The general response to stress is sympathetic overactivity due to the stimulation of sympathetic centre in the hypothalamus, liberating the adreno-medullary hormones — adrenaline and noradrenaline. Sympathetic stimulation has an inhibitory effect on gastric activity[9].

When the body temperature of the dogs is raised by administering TAB vaccine, there is a decrease in the volume of gastric secretion, and its free and total acidity (Table 2). These results are not of much statistical significance, perhaps due to the mild degree of pyrexia employed.

TABLE 1 — EFFECT OF EXPOSURE TO COLD AND WARMTH ON HISTAMINE INDUCED GASTRIC SECRETION OF DOG

Sl No.		Environmental temp. °C.	Gastric secretion Vol.* ml.	Free acidity†	Total acidity†
		Exposure to cold			
1	BE	33	24	122	135
	AE	29	20	115	130
2	BE	33	24	121	135
	AE	29	22	120	130
3	BE	32	23	124	135
	AE	28	21	121	121
4	BE	33	22	122	130
	AE	29	20	118	127
5	BE	32	20	118	126
	AE	28	19	110	125
	Mean BE	32·6 ±0·3	22·6 ±0·7	121·4 ±0·9	132·2 ±1·6
	AE	28·6 ±0·3	20·4 ±0·5	116·8 ±1·9	128·6 ±1·7
		Exposure to warmth			
1	BE	33	26	126	140
	AE	41	23	120	135
2	BE	28	25	124	132
	AE	36	22	123	130
3	BE	32	23	121	139
	AE	40	21	110	121
4	BE	27	22	126	135
	AE	35	20	125	133
5	BE	32	21	117	133
	AE	40	17	110	120
	Mean BE	30·4 ±1·2	23·4 ±0·9	122·8 ±1·1	135·8 ±1·5
	AE	38·4 ±1·2	20·6 ±1·0	117·6 ±3·4	127·8 ±1·7

BE, before exposure; and AE, after exposure.
*t values are highly significant at 5 per cent level.
†Values expressed in m.eq. NaOH/litre.

TABLE 2 — EFFECT OF PYREXIA ON HISTAMINE INDUCED GASTRIC SECRETION OF DOG

Sl No.		Rectal temp. °C.	Gastric secretion Vol. ml.	Free acidity*	Total acidity*
1	BP	39·5	40	115	122
	AP	40·5	30	108	120
2	BP	39·0	20	109	119
	AP	40·0	14	101	110
3	BP	39·5	37	110	118
	AP	40·5	25	109	115
4	BP	39·0	16	82	94
	AP	40·0	10	84	95
5	BP	39·0	22	105	110
	AP	40·0	12	96	102
6	BP	39·0	15	80	89
	AP	40·0	10	72	82
7	BP	39·0	22	102	110
	AP	40·0	16	90	96
8	BP	39·0	15	89	96
	AP	40·0	10	84	88
	Mean BP	39·1 ±0·4	23·4 ±3·4	98·0 ±4·7	107·0 ±5·2
	AP	40·1 ±0·5	15·9 ±2·6	93·0 ±4·3	101·0 ±4·7

BP, before pyrexia; and AP, after pyrexia.
*Values expressed in m.eq. NaOH/litre.

When the body temperature of the dogs is raised by administering TAB vaccine, there is a decrease in the volume of gastric secretion, and its free and total acidity (Table 2). These results are not of much statistical significance, perhaps due to the mild degree of pyrexia employed.

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Observations on Infectious Dropsy of Indian Carps & Its Experimental Induction

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Infectious dropsy is a serious epidemical disease affecting some of the major carps cultured in India. The main symptoms of the infection are accumulation of a fluid inside the body cavity, scale protrusion, exophthalmus and inflammation of intestines. The pathogenic bacterium responsible for the disease has been tentatively identified as *Aeromonas* sp. The results of experiments conducted on artificial induction of the disease indicate that by inoculating a pure culture of the bacteria, the disease can be produced on normal fingerlings of *Catla catla, Labeo rohita* and *Cirrhina mrigala*.

DROPSY disease is an abnormal accumulation of fluid in the body of an animal and this may either affect the whole body or be localized in some organs or tissues. Infectious fish dropsy of European carp has been responsible for severe epidemics in temperate areas[1,2]. The present author has come across epidemics of dropsy in many stocking tanks in West Bengal State. Among the major carps cultured, *Catla catla* has been observed to be the most susceptible to this infection, followed by *Cirrhina mrigala* and *Labeo rohita* in the order mentioned. There have been instances of entire population of these fishes getting wiped out by the epidemic. To cite just one instance, in one jheel near Barrackpore, the annual loss due to the disease was estimated as about 7·5 metric tonnes of fish. The dropsy epidemic is generally seasonal in appearance and makes its presence known immediately after the winter season, when the temperature of the water rises. In view of the fact that very little work has been done on epidemic diseases of fishes in India and because of the extreme importance of dropsy in fish culture, detailed investigations on the subject have been taken up. The present account deals with the preliminary observations made on the disease and its artificial induction. The specimens used for the studies were obtained from stocking ponds and tanks in and around Barrackpore and Calcutta.

Symptoms of the disease — The disease can be recognized by an abnormal and rounded bulging of the belly of the fish due to accumulation of a greyish fluid inside the body cavity and an associated flabbiness of the skin. Scale protrusion, i.e. a condition in which the scales loose their firmness of attachment and either stick out or fall away, is another common symptom. Many of the affected catla and mrigal show exophthalmus, i.e. a condition in which the eyes swell considerably and bulge out[2]. Few ulcers may be noticed in some specimens. Dropsy-infected fish collected from stocking tanks in the 'Dhapa' area (sewage dump) of Calcutta always showed the presence of small ulcers. As in the case of the dropsy of European carp[3], the intestines of catla, rohu and mirgal affected by the disease have been found to be inflamed in most of the specimens. However, no infections of liver and kidneys have been observed so far. Presence of ecchymoses (red spots on the body) is also a secondary symptom of the disease. The next stage of the infection is a general septicaemia as in *Cyprinus carpio*[4]. Evidences gathered tend to indicate that death of the fish generally takes place at this stage of the infection.

Etiology — Microbiological observations made using standard methods of tissue culture[5] have indicated that the etiological agent of dropsy in Indian carps is a bacterium which has been tentatively identified as *Aeromonas* sp. The bacteria were isolated from the abdominal fluid as well as affected tissues. In the case of dropsy in *Cyprinus carpio*, the pathogenic bacterium was first considered as *Pseudomonas punctata*[6], but later on was identified as *Aeromonas liquefaciens*[1,4]. Details of the biological characteristics of the organism will be described elsewhere.

Experimental induction of the disease — Since the tissue culture experiments indicated that bacteria are responsible for the disease, experiments were set up to see whether a pure culture of the bacteria, when inoculated, will be able to produce the disease conditions in normal specimens and also to recover the pathogenic organism from the experimentally infected tissues.

For artificial induction, two methods were tried. In the first, inocula were prepared from pure cultures according to standard methods[5] and injected into normal, healthy fingerlings. The cultures were always made using the fluid present in the abdominal cavity of infected *C. catla* specimens. The diluent used was either physiological saline or distilled water. Suspensions were obtained by scraping the growths on agar slopes. General check of purity was always carried out by culturing 0·1 ml. of the final inoculum on agar medium. The modes of inoculation tried were subcutaneous and intramuscular. The site of inoculation was always disinfected with alcohol and the instruments used were properly sterilized in boiling water.

The results obtained from six sets of experiments are summarized in Table 1. The determination of pathogenicity was made in each experiment on three fingerlings at a time. When reasonable evidence of

TABLE 1 — ARTIFICIAL INDUCTION OF FISH DROPSY

(*Volume of inoculum*, 0·2 *ml.*)

Fish	Subcutaneous injection				Intramuscular injection			
	1	2	3	Control	1	2	3	Control
Catla catla	+	+	−	−	+	+	−	−
	−	+	+	−	+	−	+	−
	−	−	+	−	+	−	+	−
Labeo rohita	−	+	+	−	−	−	−	−
	−	+	−	−	−	+	−	−
	+	−	−	−	−	−	+	−
Cirrhina mrigala	−	−	+	−	+	−	+	−
	+	+	−	−	+	−	+	−
	−	+	+	−	−	−	−	−

+, positive reaction; −, negative reaction.

infection could be seen, the result was considered as positive. The experiments provide sufficient proof to show that the bacteria are pathogenic to all the three species of fish studied. The order of susceptibility of the fish to the pathogen has been found to be more or less similar to that observed under natural conditions, viz. catla, mrigal and rohu.

In the second method tried, artificial inducement was attempted by contaminating the water in which the normal fingerlings were kept and also by giving food smeared with the growths on agar. In a total of six trials made in each case, only two specimens showed at least slight indications of the disease. Incidentally, it may be mentioned that one gold-fish kept in an aquarium in the laboratory developed symptoms of dropsy by accidental contamination of the water with the fluid from an infected catla. Autopsy of the specimen showed the presence of abdominal fluid and inflammation of intestine.

Cultures prepared from the affected tissues of the artificially infected fish showed the presence of bacteria identical to the inoculated ones.

Remarks — In recent years there has been considerable controversy about the exact origin of dropsy disease in *Cyprinus carpio*. German and Russian investigators have gathered evidences to show that although the bacteria have an important role in the development of dropsical conditions, a virus is the primary cause of the disease[1,2]. The present observations made on the infectious dropsy in Indian carps show that the causative bacterium can be isolated in pure cultures made from the abdominal fluid and other affected tissues. It has also been possible to artificially induce the infection by inoculation and to re-isolate the pathogen. These observations appear to satisfy Koch's postulates[6] for verifying pathogenicity of bacteria. However, the results must be treated as preliminary only, because there is no definite assurance that the inocula did not contain some virus also. Further investigations are expected to throw more light on this aspect.

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Effect of Gibberellic Acid on Vegetative Growth & Total Alkaloids of *Rauvolfia serpentina* Benth.

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Gibberellic acid solutions of different concentrations (25-200 p.p.m.), when sprayed on the seedlings of *R. serpentina* at preflowering stage, have been found to markedly enhance the rate of growth of all parts of the plant, the effect being most pronounced on the stem. The development of total alkaloids in the roots, however, showed a downward trend.

THE action of gibberellic acid (G.A.) in causing elongation of plant cells is well known. Dwarf plants tend to elongate when G.A. in suitable concentrations is applied. Considerable work has been done on the effect of G.A. on the vegetative growth of many horticultural and agricultural plants, but little work has been reported on the medicinal plants.

The present study was undertaken to examine the effect of G.A. on the total alkaloidal content and vegetative growth of *Rauvolfia serpentina* Benth., whose cultivation is being attempted at various places.

Gibberellic acid (gibberellin A_3), obtained through the courtesy of the Imperial Chemical Industries, England, was dissolved in minimum quantity of ethanol and the alcoholic solution diluted with distilled water to make solutions of concentrations 25, 50, 100 and 200 p.p.m. Seeds of *R. serpentina* obtained from the Ramnath Chopra Garden of Medicinal Plants, Jammu, were sown in seed beds in the second week

of July 1959 and the seedlings were transplanted in pots after about six weeks, when they had attained a height of 8-10 cm. and about 5-7 young leaves had appeared. G.A. solution was sprayed with a hand atomizer on the aerial parts at the preflowering stage of the seedlings two weeks after transplanting, and spraying was repeated after one week.

The effect of G.A. on the plants could be observed three to four days after its application. The internodes of the stem and midrib of new leaves (including the lamina) showed marked elongation as compared with those of the controls. Linear measurements of the aerial shoots of the plants were made two weeks after the second treatment and the results are given in Table 1.

The terminal internodes in the seedlings treated with 100-200 p.p.m. of G.A. elongated abnormally to give a vine-like appearance; lower concentrations produced steady elongation of subapical internodes. The peduncles of the inflorescence exhibited more marked elongation than the individual pedicels of the flowers in the treated plants as compared with those of control plants. The leaves of the treated plants, particularly the newly developed ones, turned pale green in colour; the intensity of chlorosis was more in plants treated with higher concentrations of G.A.

The G.A. was observed to influence the linear growth of the plants for three to four weeks only, after which the process of extra elongation ceased, which indicates that G.A. has no cumulative effect. The treated plants also exhibited an early setting of seeds, as compared with those of the control. With the onset of autumn the leaves, flowers and fruits were shed and the plants remained dormant for 12-15 weeks, till the next spring, when the shoots bore new leaves and flowered in June 1960. No well-marked floral differences were carried over to the second year of growth of the plants, except for general elongation of the main shoots, varying with the concentration of G.A. in the previous season. All the treated plants showed normal flowering in the second year of growth and there was normal setting of seed.

The plants with the entire root system were removed from the pots during the autumn of 1960, i.e. about 14 months after the date of planting. On comparing the development of root system, it was observed that the treated plants developed larger number of secondary roots than the main primary roots. The dry weight in the primary and secondary roots in the treated plants also exhibited an increase as compared to the control plants. The formation of fibrous roots which occurred in all the treated and untreated plants was, however, not much influenced by the G.A. treatment.

Data regarding the total alkaloids contents of the roots, determined by the official method described in *Indian Pharmacopoeia* (1954) given in Table 1, show that G.A. at all the concentrations studied lowered the percentage of total alkaloids in the roots as compared with the control plants, the decrease being more marked in the plants treated with higher concentrations of G.A. Similar decreases in the alkaloidal content of the roots of *Hyoscyamus niger*[1], *Atropa belladonna*[2] and *Datura stramonium*[3,4] on treatment with G.A. have been reported in literature.

TABLE 1 — STEM LENGTH AND ALKALOIDAL CONTENT OF ROOTS OF *R. SERPENTINA* TREATED WITH G.A.

(*Values are the averages of five estimations*)

Conc. of G.A. *p.p.m.*	Length of stem, *cm.*		Alkaloidal content of roots % (*wt/wt*)
	Before application	15 days after treatment	
Control	7·0	9·0	1·78
25	8·0	13·5	1·76
50	6·0	12·0	1·80
100	7·0	13·5	1·50
200	6·0	15·0	1·40

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A Simple Method of Determining Leaf Area in *Mentha arvensis* L.

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A rapid and simple method for estimating the leaf area of *Mentha arvensis* by measuring the length and breadth of the leaf has been described. The nature of relationship that exists between the product of length and breadth of leaf and the leaf area has been statistically examined and a regression equation has been worked out. With the help of this equation, it is possible to estimate the leaf area of a large number of samples in the field.

MEASUREMENT of leaf area can be accomplished by direct methods using a planimeter or a squared paper. Although accurate, these methods

involve the plucking of leaves from the plant. However, if rapid estimations are desired and the plucking of leaves has to be avoided, indirect methods, involving linear measurements and computation of the leaf area, have to be employed. A number of workers have taken the geometry of leaves into consideration for selecting suitable axes of measurement[1-3]. Another group of workers[4-7] used the constant k in the equation $A = kLB$ (A = leaf area, L = length of leaf and B = breadth of leaf) in different species. This equation has been utilized in the case of leaves of cereal plants which conform to a definite shape. The various approaches to this problem have been reviewed recently by Kemp[8] who has pointed out that, in these approaches, the statistical assumptions and their validity have not been discussed.

A technique which is fairly rapid and at the same time requires a simple calculation to estimate the leaf area by taking only two measurements, viz. length and breadth of the leaves at their maxima, has been developed and applied to the leaves of *Mentha arvensis*.

Leaves (105) of different ages and sizes in eleven random groups (not size groups) were studied. Leaf area was determined by squared paper method and the length and breadth of the leaves were noted. The values for leaf area ranged between 2·65 and 13·66 sq. cm. Maximum breadth of the leaves ranged between 1·2 and 3·2 cm., and maximum length between 3·1 and 6·7 cm.

Correlation coefficients (r values) were worked out between leaf area (A) and product of length and breadth ($L \times B = x$) separately for the eleven groups and a high degree of association was found between these two measurements (r = 0·8-0·9 significant at 1 per cent level in all cases). Homogeneity of the groups from which r values were calculated was tested by using the following formula[9]:

$$\chi^2 = \Sigma[(Z - Zm)^2(n-3)]$$

where Z = transformed value of $r = \frac{1}{2} \log_e \frac{1+r}{1-r}$, Zm, average of Z values, and n, the number of pairs from which r values were calculated. The groups were found to be homogeneous in nature. A pooled estimate of r (corresponding to Zm value) was found to be 0·9425.

Attempts to arrive at a constant k employing the equation $A = kLB$ did not yield any fruitful result as the value of k varied between 0·59 and 0·77, which was, however, independent of the leaf size. Therefore, the exact relationship between leaf area and the product of length and breadth was established by working out the linear regression coefficient b of Y (leaf area) on x (product of length and breadth). The linear relationship was examined by performing the t-test for b value against its standard error[10]. The b value was found to be significant and, therefore, it could be asserted with reasonable confidence that there is a definite relationship between Y and x, which may be stated as follows:

$$Y = 0{\cdot}65 + 0{\cdot}594x$$

With the help of this equation a standard chart could be prepared as a ready reckoner for obtaining the estimated leaf area against any observed $L \times B$ value.

The usefulness of such charts are obvious, especially under field conditions, when a large number of observations have to be taken in a relatively short time.

Thanks are due to Shri D. N. Dhar and Shri N. K. Gupta for their help in taking observations and computations.

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